Final Report

ER-1223: Ecological Risk Assessment of Ammonium Perchlorate on Fish, Amphibians, and Small Mammals

February 2003

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A FINAL REPORT

ENTITLED

UPTAKE AND ELIMINATION OF PERCHLORATE IN AMERICAN BULLFROG LARVAE, *RANA CATESBEIANA*

STUDY NUMBER:	RANA-01-01
SPONSOR:	Strategic Environmental Research and Development Program 1155 Herndon Parkway, Suite 900 Herndon, Virginia 20170
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University Texas Tech University Health Sciences Center Box 41163 Lubbock, TX 79409-1163
TEST SITE:	Department of Biological Sciences –**AP** Texas Tech University Box 4-3131 Lubbock, Texas 79409-3131
ANALYTICAL TEST SITE:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Center Box 41163 Lubbock, Texas 79409-1163
RESEARCH INITIATION:	November 4, 2001
RESEARCH COMPLETION:	December 22, 2001

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GOOD LABORATORIES PRACTICES STATEMENT

Project RANA-01-01, entitled " Uptake and elimination of perchlorate in American bullfrog larvae, *Rana* catesbeiana", was performed whenever possible in the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Noted exceptions are as follows:

Submitted By:

James A. Carr, Ph.D

March 28, 2002
Date

QUALITY ASSURANCE STATEMENT

This study was conducted under the Institute of Environmental and Human Health's Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Any changes in protocol and SOPs were documented in writing and signed by the study director.

The Quality Assurance Officer verbally notified the Study Director of all findings at the time of the inspection. Written audit reports were also submitted to the Study Director and Test Facility Management. Audits were performed for the following phases of the project:

Auditable Research Phase / Activity	Audit Dates		Date written report submitted to Study Director	Date written report submitted to Management
	Start	End		
Final Report and Raw Data Review	03/04/02	03/20/02		

Submitted By:

Ryan Bounds
Quality Assurance Manager

3/28/02
Date

1. DESCRIPTIVE STUDY TITLE:
Uptake and elimination of perchlorate in American bullfrog larvae, *Rana* catesbeiana.

2. STUDY NUMBER: RANA-01-01

3. SPONSOR: Strategic Environmental Research
and Development Program (SERDP)
1155 Herndon Parkway, Suite 900
Herndon, Virginia 20170

4. TESTING FACILITY NAME & ADDRESS:
Department of Biological Sciences –**AP**
Texas Tech University
Box 4-3131
Lubbock, TX 79409-3131

5. EXPERIMENTAL START & TERMINATION DATES:
Start Date: November 4, 2001
Termination Date: December 22, 2001

6. KEY PERSONNEL:
James A. Carr, Testing Facility Manager
Wanda L. Goleman, Study Director
Todd Anderson, Analytical Chemist
Ryan Bounds, Quality Assurance Officer
Ron Kendall, Principal Investigator

7. STUDY SUMMARY
Larval bullfrogs were exposed to 117 ppm ammonium perchlorate for 96 hr. Whole-body perchlorate concentrations were determined using analytical methods developed by Anderson and Wu (2002). Whole-body perchlorate concentrations increased linearly up to 96 hrs during exposure. Whole-body perchlorate content was still significantly elevated 48 hrs after transfer to plain water. This suggests that perchlorate elimination in tadpoles occurs relatively slowly. Precisely where perchlorate is sequestered in tadpoles is unclear, although data in lab rodents indicate that the thyroid gland may sequester perchlorate. Additional work is needed to determine tissue-specific uptake and elimination of perchlorate in tadpoles.

8. STUDY OBJECTIVES / PURPOSE:
To determine the rate of AP uptake and elimination in *Rana catesbeiana* (American bullfrog) tadpoles after exposure to a sub-lethal concentration of AP.

9. TEST MATERIALS:
Test Chemical name: Ammonium Perchlorate
CAS number: 7790-98-9

Characterization: 99.999% pure, found to be stable in reverse osmosis water for 109 d
Source: Aldrich Chemical Company
Reference Chemical name: aged, dechlorinated tap water
CAS number: not applicable
Characterization: tap water which has been aged at least 48 h
Source: Lubbock water supply

10. JUSTIFICATION OF TEST SYSTEM

Perchlorate occurs in ground and surface waters in 44 states in the USA, principally as a result of ammonium perchlorate (AP) discharge from rocket fuel manufacturing facilities or from the demilitarization of missiles (Urbansky, 1998). AP is highly water-soluble and, because reduction of the central chlorine atom occurs very slowly, AP can persist in the environment for decades (Urbansky, 1998).

Ionic perchlorate competitively inhibits thyroidal iodide uptake in mammals (Wolff, 1998) and also disrupts normal thyroid accumulation of iodide in many animals including fishes and amphibians (Miranda et al., 1996; Manzon and Youson, 1997). Sustained exposure to perchlorate leads to hypertrophy and hyperplasia of follicular cells, resulting in an increased thyroid weight (Siglin et al., 2000).

Because of the important role played by thyroid hormones in animal development and reproduction, disruption of thyroid function is likely to lead to serious impairments in growth, reproductive fitness, and consequently, amphibian and wildlife population stability as well as human health.

Rana catesbeiana tadpoles will be used since *R. catesbeiana* is a native species of the LHAAP area.

11. TEST ANIMALS (Where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test system):
Species: American Bullfrog, *Rana catesbeiana*
Strain: wild type
Age: prometamorphic tadpoles (Taylor-Kollros stage XV-XVII)
Number: Approximately 300
Source: Carolina Biological Supply

12. PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Each tank was labeled as indicated in section 5.5 of SOP ET-1-02, which includes genus and species name, common name, project number, and start date, date of receipt (if applicable), sex of the individuals (if appropriate), date eggs were laid and hatched (if applicable), date and time of initial exposure, solution content and concentration, date of removal from test solution, if applicable, and the name of the person responsible for animal care.

13. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

In phase I, we determined the rate of whole body perchlorate uptake by prometamorphic (Taylor and Kollros [TK] stage XV-XVII) *R. catesbeiana* larvae. Larvae

were exposed to perchlorate (117 ppm) or untreated water for 0, 24, 48, 72, or 96 h. A total of three trials were conducted with approximately 50 larvae exposed during each trial.

In the second phase, we determined the rate of elimination of perchlorate by prometamorphic (TK stage XV-XVII) *R. catesbeiana* larvae after exposure to sub-lethal concentrations. Prometamorphic *R. catesbeiana* larvae were exposed to perchlorate (117 ppm) or untreated water for 96 h, followed by a nontreatment recovery period of 0, 24, 48, 72, or 96 h. A total of three trials were conducted with approximately 50 larvae exposed during each trial. A study total of approximately 300 larvae were used.

14. METHODS:

In phase I, approximately 10 *Rana* larvae, TK stage XV-XVII (Taylor and Kollros 1946), were exposed to 117 ppm AP (dissolved in aged tap water) or untreated water for 0, 24, 48, 72, or 96 h to determine the rate of perchlorate uptake. Each treatment was performed in triplicate.

In phase II, approximately 10 *Rana* larvae, TK stage XV-XVII, were exposed to 117 ppm AP for 96 h, with tissue collections made at 0, 24, 48, 72, and 96 h after removal from test solution for determination of the rate of perchlorate elimination. Each treatment was performed in triplicate.

This gave approximately 30 larvae per treatment, for a study total of approximately 300 larvae. The AP concentration used represents a high sublethal concentration to ensure maximum uptake.

14.1 Test System Acquisition, Quarantine, Acclimation

Approximately 300 *Rana catesbeiana* larvae, TK stage XV-XVII, were obtained from Carolina Biological Supply. They were maintained in 45-L glass tanks containing 18 L of aged, dechlorinated tap water for 1-2 d prior to initiation of study at approximately 24 $\pm$ 2 °C on a 12L: 12D light regimen. Please refer to SOP AF-1-05 for details on *Rana* husbandry.

14.2 Test Condition Establishment

Acclimated prometamorphic larvae (TK stage XV-XVII) were used. They were counted into groups of 10 prior to placement into test or control solutions for a total of 30 larvae per treatment. Each group was added to each tank containing a single test concentration of AP or control solution within the set time frame. Each tank was labeled as indicated in section 5.5 of SOP ET-1-02, which includes genus and species name, common name, project number, and start date, date of receipt (if applicable), sex of the individuals (if appropriate), date eggs were laid and hatched (if applicable), date and time of initial exposure, solution content and concentration, date of removal from test solution (if applicable), and the name of the person responsible for animal care.

14.3 Test Material Application

Test material was premixed to appropriate concentrations and added to the appropriately labeled glass aquaria (see section 15.2). Larvae were added to 21 L glass aquaria

containing 8 L of test or reference solution. A 50% solution change containing the identical concentration of test substance was performed daily.

Rates/concentrations: 0, 117 ppm AP.

Frequency: Constant exposure for 0 to 96 h

Route/Method of Application:
Larvae were exposed to AP in the tank medium. Larvae were maintained in 8 L of the test or control solution in 21 L glass aquaria maintained at 24 °C ± 2 °C for 0, 24, 48, 72, or 96 h for uptake studies, and 96 h for elimination studies. Method of application was immersion. Route of exposure was via dermal, oral, and respiratory exposure as the chemical was in the aquaria medium.

Justification for Exposure Route: *Rana* are fully aquatic as larvae and maintain a semi-aquatic lifestyle as adults.

Exposure Verification: Samples of test and reference solutions were analyzed for perchlorate content (TIEHH SOP AC-2-11).

14.4 Test System Observation

Beginning on the day of initial exposure, % mortality (#dead larvae/total) % deformities (bent tails, asymmetric tails/total), edema (distention of body with fluid/total), and abnormal swimming (# showing abnormal swimming/total) were noted daily for each test and reference solution. Dead animals were removed and preserved in 10% neutral-buffered formalin (NBF). Taylor & Kollros (1946) stage was determined, and snout-vent length, hind-limb length and total length will be measured prior to exposure.

14.5 Animal Sacrifice and Sample Collections

Sample collections were performed at 0, 24, 48, 72, and 96 h. Larvae were staged, measured, and weighed, (see section 15.4), euthanized in 3-aminobenzoic acid ethyl ester (MS-222, 1g/L, SOP AF-3-03), rinsed in distilled water and frozen on dry ice for subsequent determination of whole body perchlorate content.

14.6 Endpoint Analysis

Percent mortality (#dead/total), % deformities (bent tails, asymmetric tails/total), edema (distention of body with fluid/total), and abnormal swimming was noted. Taylor & Kollros (TK stage, 1946) stage, weight, and measurements (see section 15.4) were recorded on completion of each phase.

15.0 STATISTICAL METHODS

Uptake and elimination rates, snout-vent length, hind limb length, total length, and body weight will be analyzed by two-way ANOVA (Treatment x Time).

16.0 PROTOCOL CHANGES / REVISIONS:

See attached change in study documentation forms.

17.0 RESULTS:

Figure one shows the results of the Phase 1 exposure in which tadpoles were exposed to AP for varying lengths of time. Whole-body perchlorate content increased linearly through the experimental period, and was significantly greater than the time 0 values at 72 and 96 hr after the initiation of the exposure. Figure 2 shows the results of the phase II experiment, where tadpoles were exposed to AP for 96 hr and then allowed varying amounts of non-treatment recovery time prior to tissue collection. Whole-body perchlorate 24 hr after transfer was considerably less (23,000 ng/g) than at 96 hr of exposure (53, 000 ng/g), but was still significantly higher than non-exposed controls. Whole-body perchlorate content decrease linearly through the recovery period and after 48 hr was not statistically different from the non-exposed controls, although there was still as much as 9,600 ng/g tissue perchlorate after 96 hr of recovery (Fig. 2). Tank and stock perchlorate concentrations were close to nominal concentrations for both experiments (Tables 1-3, Fig. 3). There were no effects of perchlorate on growth or development of tadpoles during the short exposure periods employed in the present study (Tables 4-5).

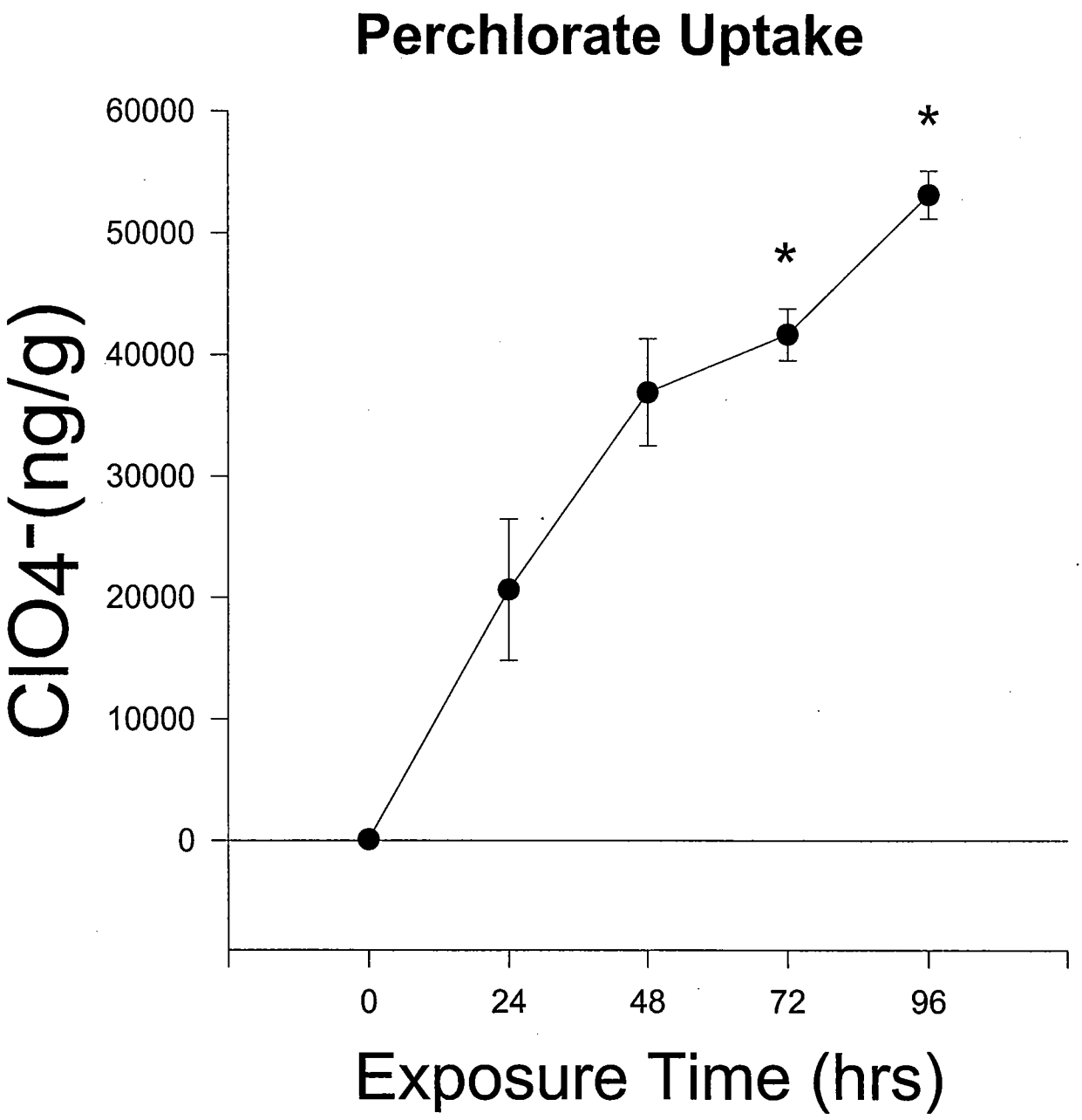

Figure 1. Whole-body perchlorate content in *R. catesbeiana* tadpoles after exposure to 117 ppm AP for varying lengths of time. * Significantly greater than timepoint 0.

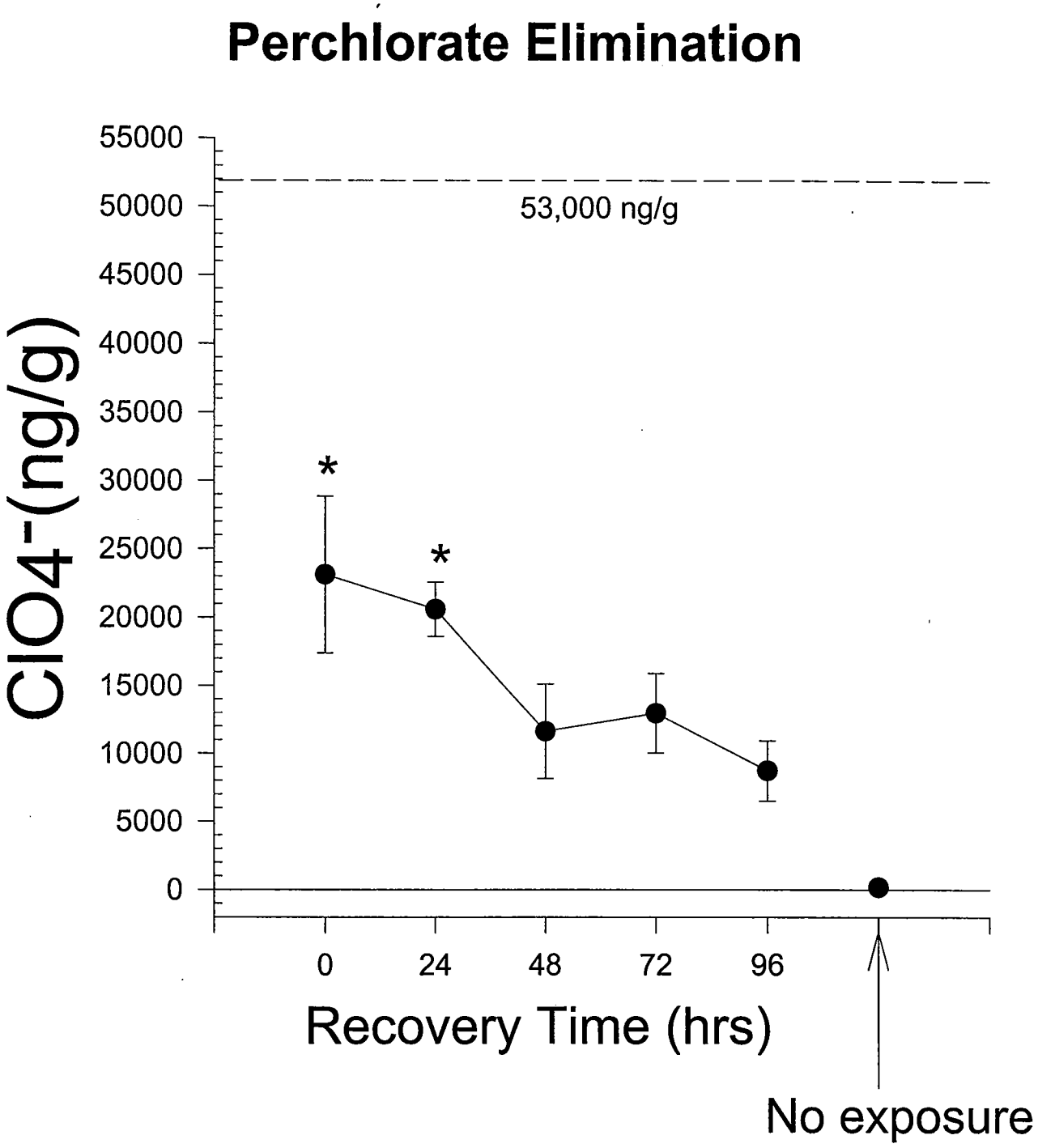

Figure 2. Whole-body perchlorate content in *R. catesbeiana* tadpoles after exposure to 117 ppm AP for 96 hr and then transfer to non-treatment recovery tanks for varying lengths of time. The dashed line indicates perchlorate whole-body levels in tadpoles at the 96 hr timepoint, prior to recovery.

* Significantly greater than non-exposed controls.

Table 1. Stock Perchlorate Solutions for Phase I.

Preparation date	Nominal perchlorate (ppb)	Actual perchlorate (ppb)
10/11/01	11750000	13074001
10/24/01	11750000	12601989
11/4/01	117000	106276
11/4/01	117000	107000

Table 2. Perchlorate Tank Concentrations for Phase I.

Tank	Date	Nominal perchlorate (ppb)	Actual Perchlorate (ppb)
RANA-1	11/5/01	117000	111976
	11/6/01	117000	112345
	11/7/01	117000	111483
	11/8/01	117000	115041
RANA-2	11/5/01	117000	119596
	11/6/01	117000	110724
	11/7/01	117000	109690
	11/8/01	117000	116024
RANA-3	11/5/01	117000	119219
	11/6/01	117000	116897
	11/7/01	117000	109690
	11/8/01	117000	113156
RANA-4	11/5/01	117000	115843
	11/6/01	117000	112414
	11/7/01	117000	109310
RANA-5	11/5/01	117000	108060
	11/6/01	117000	108138
	11/7/01	117000	105896
RANA-6	11/5/01	117000	112484
	11/6/01	117000	10986
	11/7/01	117000	107172
RANA-7	11/5/01	117000	118105
	11/6/01	117000	108103
RANA-8	11/5/01	117000	120562
	11/6/01	117000	108034
RANA-9	11/5/01	117000	114696
	11/6/01	117000	108896
RANA-10	11/5/01	117000	133736
RANA-11	11/5/01	117000	110977
RANA-12	11/5/01	117000	106389
RANA-13	11/5/01	0	0
RANA-14	11/5/01	0	0
RANA-15	11/5/01	0	0

Table 3. Stock Perchlorate Solutions for Phase II.

Preparation date	Nominal perchlorate (ppb)	Actual perchlorate (ppb)
12/14/01	117000	209291
12/14/01	117000	102985

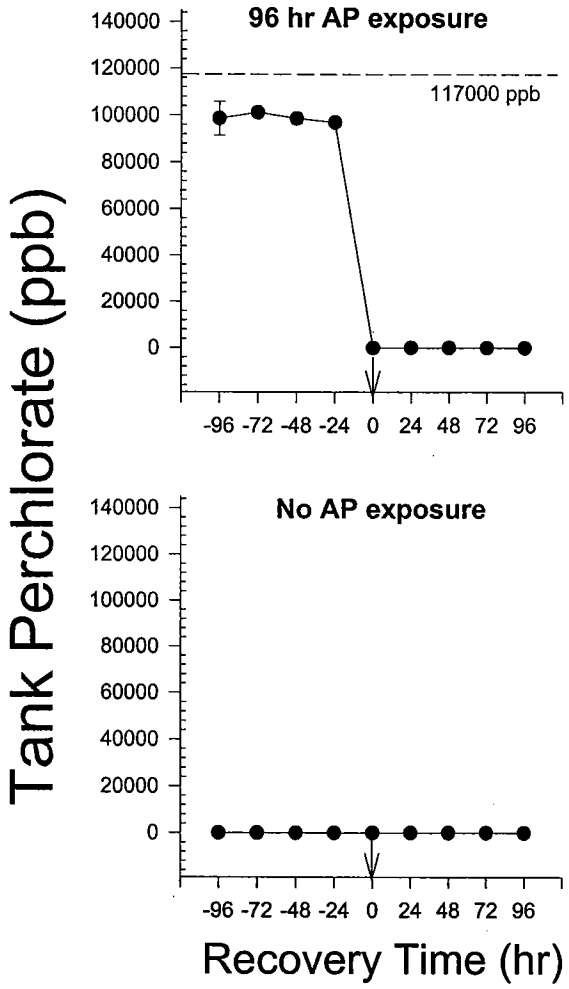

Figure 3. Tank perchlorate content during and after exposure to 117 ppm AP for 96 hr. Arrows indicate transfer of tadpoles to non-treatment recovery tanks.

Table 4. Developmental Stage, Length (mm), and Weight (g) of *R. catesbeiana* tadpoles used in Phase I.

Exposure Time	TK Stage*	SVL** (mm)	Total Length (mm)	HLL*** (mm)	Weight (gm)
96 hr	IV	28.2 ± 0.6	65.6 ± 1.0	1.6 ± 0.1	3.2 ± 0.2
72 hr	VI	28.0 ± 0.5	68.0 ± 1.3	2.0 ± 0.2	3.6 ± 0.2
48 hr	V	28.3 ± 0.5	69.0 ± 1.3	1.9 ± 0.2	3.6 ± 0.2
24 hr	V	26.2 ± 0.3	64.7 ± 1.0	1.4 ± 0.1	3.0 ± 0.1
0 hr	V	27.6 ± 0.6	67.5 ± 1.2	1.5 ± 0.1	3.4 ± 0.2

All values are mean + S.E.
* TK Stage (Taylor-Kollros stage; Taylor and Kollros, 1946)
** SVL (snout-vent length)
*** HLL (hindlimb length)

Table 5. Developmental Stage, Length (mm), and Weight (g) of *R. catesbeiana* tadpoles used in Phase II.

Elimination Time	TK Stage*	SVL** (mm)	Total Length (mm)	HLL*** (mm)	Weight (gm)
96 hr	IV	24.0 ± 0.8	56.9 ± 1.9	1.1 ± 0.2	2.2 ± 0.2
72 hr	IV	24.1 ± 1.0	57.1 ± 2.3	1.3 ± 0.2	2.1 ± 0.2
48 hr	IV	23.0 ± 1.0	53.3 ± 2.4	0.9 ± 0.2	1.9 ± 0.3
24 hr	III	20.9 ± 0.6	48.1 ± 1.5	0.6 ± 0.1	1.3 ± 0.1
0 hr	III	20.9 ± 0.6	49.1 ± 1.7	0.7 ± 0.1	1.4 ± 0.1
No Exposure	IV	22.1 ± 0.8	51.2 ± 1.8	0.9 ± 0.1	1.7 ± 0.2

All values are mean + S.E.
* TK Stage (Taylor-Kollros stage; Taylor and Kollros, 1946)
** SVL (snout-vent length)
*** HLL (hindlimb length)

18.0 DISCUSSION

To our knowledge this is the first study to investigate perchlorate uptake and elimination in any amphibian species. Our data suggest that larval bullfrogs accumulate significant amounts of perchlorate from the surrounding medium. The greatest amount of perchlorate uptake was observed in tadpoles exposed to perchlorate for 96 hr, the longest time point examined in the present study. Perchlorate uptake was still linear at 96 hr, suggesting that uptake would likely have continued beyond this time point if the experiment had been allowed to continue.

Within 24 hours after transfer to a non-treatment recovery tank, perchlorate levels had decreased approximately by half, but were still significantly greater at 24 hr and 48 hr of recovery than whole-body perchlorate in non-exposed controls. This suggests that perchlorate elimination in tadpoles is somewhat slow, requiring days. Exactly where perchlorate is being stored in these animals is unclear at this time, although tissue-specific perchlorate uptake will be the examined in future experiments. In rats, there is conflicting literature regarding the ability of the thyroid gland to accumulate perchlorate (Lewitus et al., 1962; Chow et al., 1969; Chow and Woodbury, 1970; Goldman and Stanbury, 1973).

The AP concentrations selected for the present study are much higher than one would expect to encounter in the environment. This concentration was selected primarily to ensure detectable perchlorate concentrations for tissue analysis, as the tissue analysis protocol is not as sensitive as the water analysis.

19.0 STUDY RECORDS AND ARCHIVE:

Study Records will be maintained at The Institute of Environmental and Human Health (TIEHH) Archive for a minimum of one year after study completion date.

20.0 REFERENCES:

Anderson, T.A., and T. H. Wu. (2002). Extraction, cleanup, and analysis of the perchlorate anion in tissue samples. Bull Environ. Contam. Toxicol. In press.

Chow, S.Y. and Woodbury, D.M. (1970). Kinetics of distribution of radioactive perchlorate in rat and guinea-pig thyroid glands. J. Endocrinol.47:207-218.

Chow, S.Y., Chang, L.R., Yen, M.S. (1969). A comparison between the uptakes of radioactive perchlorate and iodide by rat and guinea-pig thyroid glands. J. Endocrinol. 45:1-8.

Goldman, S.J. and Stanbury, J.B. (1973). The metabolism of perchlorate in the rat. Endocrinology 92:1536-1538.

Lewitus, Z., Guttmann, S., and Anbar M. (1962). Effect of thyroid-stimulating hormone (TSH) on the accumulation of perchlorate and fluoroborate ions in the thyroid glands of rats. Endocrinology 70:295-297.

Manzon, R.G. and Youson, J.H. (1997). The effects of exogenous thyroxine (T_4) on triiodothyronine (T_3), in the presence or absence of potassium perchlorate, on the incidence of metamorphosis and on serum T_4 and T_3 concentrations in larval sea lampreys (*Petromyzon marinus* L). Gen. Comp. Endocrinol. 106: 211-220.

Miranda, L.A., Pisano, A. and Casco, V. (1996). Ultrastructural study of thyroid glands

of *Bufo arenarum* larvae kept in potassium perchlorate solution. Biocell 20: 147-153.

Siglin, J.C., Mattie, D.R., Dodd, D.E., Hildebrandt, P.K., Baker, W.H. (2000). A 90-day drinking water toxicity study in rats of the environmental contaminant ammonium perchlorate. Toxicol. Sci. 57:61-74.

Taylor, A.C. and Kollros, J..J. (1946). Stages in the normal development of *Rana pipens* larvae. Anat. Rec. 94: 7-23.

Urbansky, E.T. (1998). Perchlorate chemistry: implications for analysis and remediation. Bioremediation J. 2: 81-95.

21.0 APPENDICES:

Study Protocol
Changes to Study Documentation

A STUDY PROTOCOL

ENTITLED

UPTAKE AND ELIMINATION OF PERCHLORATE IN AMERICAN BULLFROG LARVAE, *RANA CATESBEIANA*

STUDY/PROTOCOL NUMBER: RANA-01-01

SPONSOR: United States Air Force
IERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

TESTING FACILITY

Name/Address: Department of Biological Sciences –**AP**
Texas Tech University
Box 4-3131
Lubbock, Texas 79409-3131

Test Facility Management: Dr. James A. Carr

Study Director: Wanda L. Goleman

PROPOSED EXPERIMENTAL
START DATE October 11, 2001

1. **DESCRIPTIVE STUDY TITLE:**
Uptake and elimination of perchlorate in American bullfrog larvae, *Rana* catesbeiana.

2. **STUDY NUMBER:** RANA-01-01

3. **SPONSOR:** United States Air Force United States Air Force
IERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

4. **TESTING FACILITY NAME & ADDRESS:**
Department of Biological Sciences –**AP**
Texas Tech University
Box 4-3131
Lubbock, TX 79409-3131

5. **PROPOSED EXPERIMENTAL START & TERMINATION DATES:**
Start Date: October 11, 2001
Termination Date: February 28, 2001

6. **KEY PERSONNEL:**
James A. Carr, Testing Facility Manager
Wanda L. Goleman, Study Director
Todd Anderson, Analytical Chemist
Ryan Bounds, Quality Assurance Officer
Ron Kendall, Principal Investigator

7. **DATED SIGNATURES:**

Wanda L. Goleman	10/11/01	Ms. Wanda L. Goleman Study Director
James Carr	10/17/01	Dr. James Carr Testing Facility Management
Ryan Bounds	10/19/01	Mr. Ryan Bounds Quality Assurance Officer
Todd Anderson	10-25-01	Dr. Todd Anderson Analytical Chemist
Ronald J. Kendall	11-8-01	Dr. Ron Kendall Principal Investigator
	28-OCT-01	Dr. Lou Chiodo Asst. Director

8. **REGULATORY COMPLIANCE STATEMENT**

Quality Control and Quality Assurance

This study will be conducted in accordance with established Quality Assurance program guidelines and in compliance, where appropriate and possible, with Good Laboratory Practice Standards (40 CFR Part 160, August 17, 1989).

Document Control Statement

This document is considered proprietary to and the Sponsor. Do not copy, quote or distribute. For access to this document or authority to release or distribute, please write to:

Dr. James A. Carr
Department of Biological Sciences
Texas Tech University
Box 4-3131
Lubbock, Texas 79409

9. **STUDY OBJECTIVES / PURPOSE:**

To determine the rate of AP uptake and elimination in *Rana catesbeiana* (American bullfrog) tadpoles after exposure to a sub-lethal concentration of AP.

10. TEST MATERIALS:

Test Chemical name: Ammonium Perchlorate
CAS number: 7790-98-9
Characterization: 99.999% pure, found to be stable in reverse osmosis water for 109 d
Source: Aldrich Chemical Company

Reference Chemical name: aged, dechlorinated tap water
CAS number: not applicable
Characterization: tap water which has been aged at least 48 h
Source: Lubbock water supply

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Perchlorate occurs in ground and surface waters in 44 states in the USA, principally as a result of ammonium perchlorate (AP) discharge from rocket fuel manufacturing facilities or from the demilitarization of missiles (Urbansky, 1998). AP is highly water-soluble and, because reduction of the central chlorine atom occurs very slowly, AP can persist in the environment for decades (Urbansky, 1998).

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Because of the important role played by thyroid hormones in animal development and reproduction, disruption of thyroid function is likely to lead to serious impairments in growth, reproductive fitness, and consequently, amphibian and wildlife population stability as well as human health.

Rana catesbeiana tadpoles will be used since *R. catesbeiana* is a native species of the LHAAP area.

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Species: American Bullfrog, *Rana catesbeiana*
Strain: wild type
Age: prometamorphic tadpoles (Taylor-Kollros stage XV-XVII)
Number: Approximately 300
Source: Carolina Biological Supply

13. PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Each tank will be labeled as indicated in section 5.5 of **DBS SOP ET-1-02**, which includes genus and species name, common name, project number, and start date, date of receipt (if applicable), sex of the individuals (if appropriate), date eggs were laid and hatched (if

applicable), date and time of initial exposure, solution content and concentration, date of removal from test solution, if applicable, and the name of the person responsible for animal care.

14. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

In phase I, we will determine the rate of whole body perchlorate uptake by prometamorphic (Taylor and Kollros [TK] stage XV-XVII) *R. catesbeiana* larvae. Larvae will be exposed to perchlorate (10^{-3} M) or untreated water for 0, 24, 48, 72, or 96 h. A total of three trials will be conducted with approximately 50 larvae exposed during each trial.

In the second phase, we will determine the rate of elimination of perchlorate by prometamorphic (TK stage XV-XVII) *R. catesbeiana* larvae after exposure to sub-lethal concentrations. Prometamorphic *R. catesbeiana* larvae will be exposed to perchlorate (10^{-3} M) or untreated water for 96 h, followed by a nontreatment recovery period of 0, 24, 48, 72, or 96 h. A total of three trials will be conducted with approximately 50 larvae exposed during each trial. A study total of approximately 300 larvae will be used.

15. METHODS:

In phase I, approximately 10 *Rana* larvae, TK stage XV-XVII (Taylor and Kollros 1946), will be exposed to one concentration of AP (dissolved in water) or untreated water for 0, 24, 48, 72, or 96 h to determine the rate of perchlorate uptake. Each treatment will be performed in triplicate.

In phase II, approximately 10 *Rana* larvae, TK stage XV-XVII, will be exposed to a like concentration of AP for 96 h, with collections made at 0, 24, 48, 72, and 96 h after removal from test solution for determination of the rate of perchlorate elimination. Each treatment will be performed in triplicate.

This will give approximately 30 larvae per treatment, for a study total of approximately 300 larvae. The AP concentration to be used represents a high sublethal concentration to ensure maximum uptake.

15.1 Test System Acquisition, Quarantine, Acclimation

Approximately 300 *Rana catesbeiana* larvae, TK stage XV-XVII, will be obtained from Carolina Biological Supply. They will be maintained in 45-L glass tanks containing 18 L of aged, dechlorinated tap water for 1-2 days prior to initiation of study at approximately 24 $\pm$ 2 °C on a 12L: 12D light regimen. Please refer to **DBS SOP AF-1-05** for details on *Rana* husbandry.

15.2 Test Condition Establishment

Acclimated prometamorphic larvae (TK stage XV-XVII) will be used. They will be counted into groups of 10 prior to placement into test or control solutions for a total of 30 larvae per treatment. Each group will be added to each tank containing a single test

concentration of AP or control solution within the set time frame. Each tank will be labeled as indicated in section 5.5 of **DBS SOP ET-1-02**, which includes genus and species name, common name, project number, and start date, date of receipt (if applicable), sex of the individuals (if appropriate), date eggs were laid and hatched (if applicable), date and time of initial exposure, solution content and concentration, date of removal from test solution (if applicable), and the name of the person responsible for animal care.

15.3 Test Material Application

Test material will be premixed to appropriate concentrations and added to the appropriately labeled glass aquaria (see section 15.2). Larvae will be added to 21 L glass aquaria containing 8 L of test or reference solution. A 50% solution change containing the identical concentration of test substance will be performed daily.

Rates/concentrations: 0, 10^{-3} M AP.

Frequency: Constant exposure for 0 to 96 h

Route/Method of Application:

Larvae will be exposed to AP in the tank medium. Larvae will be maintained in 8 L of the test or control solution in 21 L glass aquaria maintained at 24 °C ± 2 °C for 0, 24, 48, 72, or 96 h for uptake studies, and 96 h for elimination studies. Method of application will be immersion. Route of exposure will be via dermal, oral, and respiratory exposure as the chemical will be in the aquaria medium.

Justification for Exposure Route: *Rana* are fully aquatic as larvae and maintain a semi-aquatic lifestyle as adults.

Exposure Verification: Samples of test and reference solutions will be analyzed for perchlorate content (**TIEHH SOP AC-2-11**).

15.4 Test System Observation

Beginning on the day of initial exposure, % mortality (#dead larvae/total) % deformities (bent tails, asymmetric tails/total), edema (distention of body with fluid/total), and abnormal swimming (# showing abnormal swimming/total) will be noted daily for each test and reference solution. Dead animals will be removed and preserved in 10% neutral-buffered formalin (NBF). Taylor & Kollros (1946) stage will be determined, and snout-vent length, hind-limb length and total length will be measured prior to exposure.

15.5 Animal Sacrifice and Sample Collections

Sample collections will be performed at 0, 24, 48, 72, and 96 h. Larvae will be staged, measured, and weighed, (see section 15.4), euthanized in 3-aminobenzoic acid ethyl ester (MS-222, 1g/L, **DBS SOP AF-3-03**), rinsed in distilled water and frozen on dry ice for subsequent determination of whole body perchlorate content.

15.6 Endpoint Analysis

Percent mortality (#dead/total), % deformities (bent tails, asymmetric tails/total), edema (distention of body with fluid/total), and abnormal swimming will be noted. Taylor & Kollros (1946) stage, weight, and measurements (see section 15.4) will be recorded on completion of each phase.

AP uptake and elimination rates will be determined based on whole body perchlorate content.

16. PROPOSED STATISTICAL METHODS

Uptake and elimination rates, snout-vent length, hind limb length, total length, and body weight will be analyzed by two-way ANOVA (Treatment x Time).

17. REPORT CONTENT/RECORDS TO BE MAINTAINED:

Records to be maintained include:

- Room temperature and water temperature, salinity, pH, ammonia, and dissolved oxygen content.
- Date, time, frequencies and amount of feedings per tank will be recorded. Number of expired larvae removed prior to termination of each study will be recorded, including each time, date, and aquarium.

Report content will also include presentation of data, interpretation, and discussion of the following end-points:

- AP uptake rate
- AP elimination rate
- Discussion of the relevance of the findings
- List of all SOPs used.
- List of all personnel.

18. RECORDS TO BE MAINTAINED / LOCATION:

The final report will be delivered to the Sponsor on or before **February 28, 2002**. Copies of all data, documentation, records, protocol information, as well as the specimens shall be sent to the Sponsor, or designated delivery point upon request (within six months of study completion). All data, the protocol and a copy of the final report shall be archived by the testing facility.

19. QUALITY ASSURANCE:

The Quality Assurance Unit will inspect the study at intervals to insure the integrity of the study. Written records will be maintained indicating but not limited to the following: date of inspection, study inspected, phase inspected, person conducting the inspection, findings and problems, recommended and taken action, and any scheduled reinspections. Any problems likely to effect study integrity shall be brought to the immediate attention of the Study Director. The Quality Assurance Unit will periodically submit written status reports on the study to management and the Study Director.

20. PROTOCOL CHANGES / REVISIONS:

All changes and/or revisions to the protocol, and the reasons therefore, shall be documented, signed and dated by the Study Director and maintained with the protocol and the Quality Assurance Unit.

21. REFERENCES:

Manzon, R.G. and Youson, J.H. (1997). The effects of exogenous thyroxine (T_4) on triiodothyronine (T_3), in the presence or absence of potassium perchlorate, on the incidence of metamorphosis and on serum T_4 and T_3 concentrations in larval sea lampreys (*Petromyzon marinus* L). *Gen. Comp. Endocrinol.* 106: 211-220.

Miranda, L.A., Pisano, A. and Casco, V. (1996). Ultrastructural study of thyroid glands of *Bufo arenarum* larvae kept in potassium perchlorate solution. *Biocell* 20: 147-153.

Siglin, J.C., Mattie, D.R., Dodd, D.E., Hildebrandt, P.K., Baker, W.H. A 90-day drinking water toxicity study in rats of the environmental contaminant ammonium perchlorate. Toxicol. Sci. 57:61-74, 2000.

Taylor, A.C. and Kollros, J.J. 1946. Stages in the normal development of *Rana pipens* larvae. Anat Rec 94: 7-23.

Urbansky, E.T. Perchlorate chemistry: implications for analysis and remediation. Bioremediation J. 2: 81-95, 1998.

Dept. of Biological Sciences (DBS)
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(806) 742-2715

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: X Amendment ____Deviation ____ Addendums

Document Reference Information
Check One: X Protocol ____ SOP ____ Other _____
Title: Uptake and Elimination of Perchlorate in American Bullfrog Larvae, *Rana catesbeiana*
Dated: October 11, 2001
Document # (if appropriate): RANA-01-01
Page #(s): 4; 5
Section #: 12; 14; 15; 15.1

Text to reference: 12. TEST ANIMALS (Where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test system):
Species: American Bullfrog, *Rana catesbeiana*
Strain: wild type
Age: prometamorphic tadpoles (Taylor-Kollros stage III – VII)
Number: Approximately 300
Source: Carolina Biological Supply
14. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL: In the second phase, we will determine the rate of elimination of perchlorate by prometamorphic (TK stage III – VII) *R. catesbeiana* larvae after exposure to sub-lethal concentrations. Prometamorphic *R. catesbeiana* larvae will be exposed to perchlorate (10^{-3} M) or untreated water for 96 h, followed by a nontreatment recovery period of 0, 24, 48, 72, or 96 h. A total of three trials will be conducted with approximately 50 larvae exposed during each trial. A study total of approximately 300 larvae will be used.
15. METHODS: In phase II, approximately 10 *Rana* larvae, TK stage III – VII, will be exposed to a like concentration of AP for 96 h, with collections made at 0, 24, 48, 72, and 96 h after removal from test solution for determination of the rate of perchlorate elimination. Each treatment will be performed in triplicate.

This will give approximately 30 larvae per treatment, for a study total of approximately 300 larvae. The AP concentration to be used represents a high sublethal concentration to ensure maximum uptake.
15.1 Test System Acquisition, Quarantine, Acclimation: Approximately 300 *Rana catesbeiana* larvae, TK stage III – VII, will be obtained from Carolina Biological Supply. They will be maintained in 45-L glass tanks containing 18 L of aged, dechlorinated tap water for 1-2 days prior to initiation of study at approximately 24 ± 2 °C on a 12L: 12D light regimen. Please refer to **DBS SOP AF-1-05** for details on *Rana* husbandry.

* Sequentially numbered in order of the date that the change is effective

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Change In Study Documentation Form

Change in Document: 12. TEST ANIMALS (Where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test system):
Species: American Bullfrog, *Rana catesbeiana*
Strain: wild type
Age: prometamorphic tadpoles (Taylor-Kollros stage III – VII)
Number: Approximately 330
Source: Carolina Biological Supply
14. EXPERIMENTAL DESIGN INCLUDING BIASCONTROL: In the second phase, we will determine the rate of elimination of perchlorate by prometamorphic (TK stage III – VII) *R. catesbeiana* larvae after exposure to sub-lethal concentrations. Prometamorphic *R. catesbeiana* larvae will be exposed to perchlorate (10^{-3} M) or untreated water for 96 h, followed by a nontreatment recovery period of 0, 24, 48, 72, or 96 h. A total of three trials will be conducted with approximately 50 larvae exposed and 10 nonexposed during each trial. A study total of approximately 330 larvae will be used.
15. METHODS: In phase II, approximately 10 *Rana* larvae, TK stage III – VII, will be exposed to a like concentration of AP or untreated water for 96 h, with collections made at 0, 24, 48, 72, and 96 h after removal from test solution for determination of the rate of perchlorate elimination. Each treatment will be performed in triplicate.

This will give approximately 30 larvae per treatment, for a study total of approximately 330 larvae. The AP concentration to be used represents a high sublethal concentration to ensure maximum uptake.
15.1 Test System Acquisition, Quarantine, Acclimation: Approximately 330 *Rana catesbeiana* larvae, TK stage III – VII, will be obtained from Carolina Biological Supply. They will be maintained in 45-L glass tanks containing 18 L of aged, dechlorinated tap water for 1-2 days prior to initiation of study at approximately 24 ± 2 °C on a 12L: 12D light regimen. Please refer to **DBS SOP AF-1-05** for details on *Rana* husbandry.

Justification and Impact on Study: 14. EXPERIMENTAL DESIGN INCLUDING BIASCONTROL: Three replicates of nonexposed larvae in Phase II will increase the total number of animals used to approximately 330.
15. METHODS: Three replicates of nonexposed larvae in Phase II will increase the total number of animals used to approximately 330.
15.1 Test System Acquisition, Quarantine, Acclimation: Three replicates of nonexposed larvae in Phase II will increase the total number of animals used to approximately 330.

Submitted by: Signature: Wanda L. Goleman Date: 12/12/01

Authorized by: Study Director: Wanda L. Goleman Date: 12/12/01

Received by: Quality Assurance Unit: Brian Bushwell Date: 3/28/02

* Sequentially numbered in order of the date that the change is effective

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Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: X Amendment ____Deviation ____ Addendums

Document Reference Information
Check One: X Protocol ____ SOP ____ Other _____
Title: Uptake and Elimination of Perchlorate in American Bullfrog Larvae, *Rana catesbeiana*
Dated: October 11, 2001
Document # (if appropriate): RANA-01-01
Page #(s): 5, 6
Section #: 15, 15.1, 15.2, 15.4
Text to reference: Section 15. Methods: In phase I, approximately 10 *Rana* larvae, TK stage XV-XVII (Taylor and Kollros 1946), will be exposed to one concentration of AP (dissolved in water) or untreated water for 0, 24, 48, 72, or 96 h to determine the rate of perchlorate uptake. Each treatment will be performed in triplicate.

In phase II, approximately 10 *Rana* larvae, TK stage XV-XVII, will be exposed to a like concentration of AP for 96 h, with collections made at 0, 24, 48, 72, and 96 h after removal from test solution for determination of the rate of perchlorate elimination. Each treatment will be performed in triplicate.
Section 15.1. Test System Acquisition, Quarantine, Acclimation: Approximately 300 *Rana catesbeiana* larvae, TK stage XV-XVII, will be obtained from Carolina Biological Supply.
Section 15.2. Test Condition Establishment: Acclimated prometamorphic larvae (TK stage XV-XVII) will be used.
Section 15.4. Test System Observation: Beginning on the day of initial exposure, % mortality (#dead larvae/total) % deformities (bent tails, asymmetric tails/total), edema (distention of body with fluid/total), and abnormal swimming (# showing abnormal swimming/total) will be noted daily for each test and reference solution. Dead animals will be removed and preserved in 10% neutral-buffered formalin (NBF). Taylor & Kollros (1946) stage will be determined, and snout-vent length, hind-limb length and total length will be measured prior to exposure.

Change in Document: Section 15. Methods: In phase I, approximately 10 *Rana* larvae, TK stage III - VII (Taylor and Kollros 1946), will be exposed to one concentration of AP (dissolved in water) or untreated water for 0, 24, 48, 72, or 96 h to determine the rate of perchlorate uptake. Each treatment will be performed in triplicate.

* Sequentially numbered in order of the date that the change is effective

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Change In Study Documentation Form

In phase II, approximately 10 *Rana* larvae, TK stage III - VII, will be exposed to a like concentration of AP for 96 h, with collections made at 0, 24, 48, 72, and 96 h after removal from test solution for determination of the rate of perchlorate elimination. Each treatment will be performed in triplicate.
Section 15.1. Test System Acquisition, Quarantine, Acclimation: Approximately 300 *Rana catesbeiana* larvae, TK stage III - VII, will be obtained from Carolina Biological Supply.
Section 15.2. Test Condition Establishment: Acclimated prometamorphic larvae (TK stage III - VII) will be used.
Section 15.4. Test System Observation: Beginning on the day of initial exposure, % mortality (#dead larvae/total) % deformities (bent tails, asymmetric tails/total), edema (distention of body with fluid/total), and abnormal swimming (# showing abnormal swimming/total) will be noted daily for each test and reference solution. Dead animals will be removed and preserved in 10% neutral-buffered formalin (NBF). The approximate range of Taylor & Kollros (1946) stages will be determined prior to exposure.

Justification and Impact on Study: Section 15. Although we were able to obtain *R. catesbeiana* larvae from Carolina Biological Supply, we could not specify a range of stages.
Section 15.1. Although we were able to obtain *R. catesbeiana* larvae from Carolina Biological Supply, we could not specify a range of stages.
Section 15.2. Although we were able to obtain *R. catesbeiana* larvae from Carolina Biological Supply, we could not specify a range of stages.
Section 15.4. Although we were able to obtain *R. catesbeiana* larvae from Carolina Biological Supply, we could not specify a range of stages. The approximate range of TK stages will be determined prior to exposure. Measurement of snout-vent length, hindlimb length, and total length prior to exposure is not necessary due to the short timeframe of the study.

Submitted by: Signature: Randa L. Coleman Date: 11/3/01
Authorized by: Study Director: Randa L. Coleman Date: 11/3/01
Received by: Quality Assurance Unit: Date: 3/19/02

* Sequentially numbered in order of the date that the change is effective

A FINAL REPORT

ENTITLED

RESPONSE OF NATIVE ADULT AND LARVAL ANURANS IN THEIR NATURAL ENVIRONMENT TO AMMONIUM PERCHLORATE CONTAMINATION: ASSESSMENT OF REPRODUCTIVE AND THYROID ENDPOINTS

STUDY NUMBER:	ANUR-01-01
SPONSOR:	Strategic Environmental Research and Development Program (SERDP) 1155 Herndon Parkway, Suite 900 Herndon, Virginia 20170
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
TESTING FACILITY:	Department of Biological Sciences –**AP** Texas Tech University Box 4-3131 Lubbock, TX 79409-3131
ANALYTICAL TEST SITE:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Center Box 41163 Lubbock, Texas 79409-1163
RESEARCH INITIATION:	June 8, 2001
RESEARCH COMPLETION:	September 30, 2001

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List of Tables and Figures

GOOD LABORATORY PRACTICES STATEMENT

Project ANUR-01-01, entitled " Response Of Native Adult And Larval Anurans In Their Natural Environment To Ammonium Perchlorate Contamination: Assessment Of Reproductive And Thyroid Endpoints ", was performed whenever possible in the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Noted exceptions are as follows:

1.
2.

Submitted By:

James A. Carr

James A. Carr, Ph.D

March 28, 2001

Date

QUALITY ASSURANCE STATEMENT

This study was conducted under the Institute of Environmental and Human Health's Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Any changes in protocol and SOPs were documented in writing and signed by the study director.

The Quality Assurance Officer verbally notified the Study Director of all findings at the time of the inspection. Written audit reports were also submitted to the Study Director and Test Facility Management. Audits were performed for the following phases of the project:

Auditable Research Phase / Activity	Audit Dates		Date written report submitted to Study Director	Date written report submitted to Management
	Start	End		
Final Report and Raw Data Review	2/22/02	3/08/02	3/19/02	3/19/02

Submitted By:

Ryan Bounds
Quality Assurance Manager

3/28/02
Date

1.0 DESCRIPTIVE STUDY TITLE:
Response of Native Adult And Larval Anurans In Their Natural Environment To Ammonium Perchlorate Contamination: Assessment Of Reproductive And Thyroid Endpoints

2.0 STUDY NUMBER:
ANUR-01-01

3.0 SPONSOR:
Strategic Environmental Research
And Development Program (SERDP)
1155 Herndon Parkway, Suite 900
Herndon, Virginia 20170

4.0 TESTING FACILITY NAME AND ADDRESS:
The Institute of Environmental and Human Health
Texas Tech University
Texas Tech University Health Sciences Center
Box 41163
Lubbock, TX 79409-1163

5.0 EXPERIMENTAL START & TERMINATION DATES:
Start Date: June 8, 2001
Termination Date: September 30, 2001

6.0 KEY PERSONNEL:
James A. Carr, Testing Facility Manager
Wanda L. Goleman, Study Director
Todd Anderson, Analytical Chemist
Ryan Bounds, Quality Assurance Officer
Ron Kendall, Principal Investigator

7.0 STUDY OBJECTIVES / PURPOSE:

To assess the response of the reproductive and thyroid axes in adult and larval anurans from natural populations inhabiting reference sites or sites with known ammonium perchlorate contamination at Longhorn Army Ammunition Plant (LHAAP).

8.0 STUDY SUMMARY:

We examined aspects of development and growth, thyroid function, and reproductive status in larval and adult frogs from reference and contaminated sites at LHAAP. We identified two possible cases of thyroid disruption in larval frogs from two contaminated areas at LHAAP. Bullfrog tadpoles collected from an AP contaminated pond had smaller hindlimb to snout-vent length ratios and collectively were at earlier developmental stages than tadpoles from a reference pond, even though the animals from both sites were of identical body length, and presumably, identical age class. Thyroids from the tadpoles collected at the reference site were larger than animals from the contaminated site. Collection of chorus frog tadpoles from an AP contaminated site revealed evidence for thyroid disruption at the histological level, with colloid depletion and thyroid follicle hypertrophy being significantly greater in the tadpoles from the contaminated site compared to the reference site. There was no evidence of thyroid disruption in any of the adult frogs collected. There were no observable gonadal abnormalities or cases of intersex in any of the adults collected. This is the first evidence of perchlorate-associated thyroid disruption in a natural amphibian population.

9.0 JUSTIFICATION OF TEST SYSTEM:

Perchlorate occurs in ground and surface waters in 44 states in the USA, principally as a result of AP discharge from rocket fuel manufacturing facilities or from the demilitarization of missiles (Urbansky, 1998). AP is highly water-soluble and, because reduction of the central chlorine atom occurs very slowly, AP can persist in the environment for decades (Urbansky, 1998).

Ionic perchlorate competitively inhibits thyroidal iodide uptake in mammals (Wolff, 1998) and also disrupts normal thyroid accumulation of iodide in nonmammals including amphibians (Miranda et al., 1996) and lampreys (Manzon and Youson, 1997). The loss of negative feedback due to decreased serum thyroxine (T_4) results in elevated blood thyroid stimulating hormone (TSH) levels and, subsequently, an increase in the height of thyroid follicular epithelial cells (Norris, 1997). Sustained exposure to perchlorate leads to hypertrophy and hyperplasia of follicular cells, resulting in an increased thyroid weight (Siglin et al., 2000).

Although perchlorate salts, principally $NaClO_4$ and $KClO_4$, have been used for years to prevent amphibian metamorphosis, the concentrations used to block metamorphosis are generally greater (250 to 1000 mg/L) than concentrations of perchlorate reported in contaminated surface and ground waters. In a recent study of surface waters and sediments at Longhorn Army Ammunition Plant (LHAAP) in East Texas, perchlorate levels as high as 31.2 ± 0.21 mg perchlorate/L were reported (Smith et al. 2001). Although the perchlorate levels found in contaminated surface waters at LHAAP and elsewhere are well below those traditionally known to prevent metamorphosis in experimental settings, the effects of environmentally relevant concentrations of AP on adult and larval thyroid function are generally unknown.

The goal of the present study was twofold. The first goal was to determine the incidence of thyroid disruption consistent with iodide deficiency in larval and adult anurans in and around LHAAP. The second goal of this study was to assess indices of reproductive activity and development in larval and adult frogs.

10.0 TEST ANIMALS:

Species: Bronze frog (*Rana clamitans clamitans*), Bullfrog (*Rana catesbeiana*) and other incidentals, such as the Southern leopard frog (*Rana utriculata*), Northern cricket frog (*Acris crepitans*) and the chorus frog (*Pseudacris triseriata*)
Strain: unknown
Age: larvae and adults
Sex: to be determined
Number: approximately 10 adults of each species, and approximately 50 larvae from each of six sites sampled 4 times each.
Source: six field sites: holding pond, holding pond reference ditch, Star Ranch pond, Harrison Bayou "catfish pond", Harrison Bayou downstream from shooting range, Building 25 C, as well as animals previously collected from these sites.

11.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Each field site was assigned a code name. Each collection site was referenced by its full name or a 4-letter abbreviation. Identified collection sites were holding pond (HOLP), Harrison Bayou "catfish pond" (HBCP), Harrison Bayou downstream (HBDS), holding pond reference ditch (HPRD), Star Ranch Pond (STAR), and building 25-C (B25C). Specimens from each field site were numbered with the code name and unique identifying number. The project number was part of the label on each specimen container.

12.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Each selected field site was sampled as follows: (1) sampling took place for approximately 1-2 hr by a minimum of two personnel; this included dipnetting for tadpoles around the circumference of ponds and on each bank of river areas; (2) we attempted to sample each site at least four times (see table 1), although the number of times each site was sampled was influenced by the presence/or absence of water at that site. At each site all anurans were captured and included in the sample, regardless of species, sex, or age.

13.0 METHODS:

Field sites were selected based on their history of perchlorate contamination (Smith et al., 2001). At each visit to the field site, we attempted to measure various compositional and physical parameters, including:

1. dissolved oxygen
2. air and water temperature
3. conductivity
4. salinity
5. pH
6. anuran species calling

These measures provided a habitat description of the field site and gave information that will be useful when considering the environmental fate of perchlorate. During each visit, a water sample was collected from the water body. Waters were collected by hand in clean 4-L glass bottles (AQ-3-02) and kept cool (i.e. in boxes and/or in shade) during transport to our laboratory where they were stored at approximately 4 θC until analyzed for perchlorate (AC-2-11).

We attempted to visit each field site 3-4 times. All animals were collected under Texas Parks and Wildlife scientific permit no.SPR1098-984. If present, approximately 10-50 tadpoles per site were collected by dip net, quickly anesthetized in 1% 3-aminobenzoic acid ethyl ester (MS-222; AF-3-03) and then fixed in 10% formalin for subsequent thyroid histopathology. When possible, blood collection was performed by cardiac puncture (AQ-3-01). This was performed on only two adult bullfrogs, which were the only frogs collected that were large enough to allow us to perform cardiac puncture according to our ACUC protocol. The animals were then returned to their capture site.

Because of the small sample size, blood from these 2 animals was not further analyzed. Approximately 6-10 adult animals from each site were used for assessment of gonadal and thyroid histopathology. After blood collection, these animals were euthanized in MS-222 and preserved in formalin for gonadal histopathology. All specimen containers were labeled. Labels included the project number, appropriate identification number, date of collection, collection site, and collector's initials.

15. ENDPOINT ANALYSIS.

Snout-vent length, developmental stage, total length and body weight were recorded. Insufficient blood plasma (due to the small size of the animals collected) collections prevented analysis of blood thyroid and reproductive hormones. Gonads of all fixed adult animals were examined by visual inspection to determine sex (as in IN-2-03, SOP in preparation). Thyroid glands were prepared using routine histological methods (IN-1-05, IN-1-06, IN-4-06, IN-4-07, IN-1-02, IN-1-01, IN-1-04). Sections were stained with hematoxylin and eosin (IN-1-04). In one instance thyroid histopathalogy (follicle cell height, thyroid gland volume) was assessed quantitatively as previously described (Goleman et al., 2002b). However, the labor intensiveness of this process prohibited the screening o the large number of samples collected. We there employed a more efficient qualitative histological screen to rapidly identify potential thyroid disruption at the histological level (Mann, 2000; Wolf, 2000). Thyroid gland activity was assessed qualitatively for evidence of hypertrophy, hyperplasia, and colloid depletion (DBS SOP IN-2-08-01). Adult gonadal tissue was assessed qualitatively for presence of oogonia and spermatozoa. Oogonial stage was determined using the staging method developed by Dumont (1978). Taylor-Kollros (TK) staging was used for staging larval Ranid frogs (Taylor and Kollros, 1946) while Gosner staging (1960) was used to stage other larval anurans. Larval frogs were identified using a key (Altig, 1970).

16. STATISTICAL METHODS

Data were analyzed using Students two-tailed t-test.

17. PROTOCOL CHANGES / REVISIONS:

See attached change in study documentation forms.

18. RESULTS:

Animals from 5 separate collection trips between April 2000 and July of 2001 were used for the current study. Of the 418 animals collected (Table 1), thyroid and reproductive analyses were performed on the 122 animals collected from sites shown in Table 2. These animals were selected because they represented both exposed and reference sites based on analysis of perchlorate levels in surface water samples. In addition, sufficient numbers of animals from these sites were collected to perform quantitative or semi-quantitative histological analyses and in some cases make direct comparisons between stage- or size matched animals from contaminated and reference sites.

Analysis of bullfrog tadpoles collected from a contaminated (HOLP) and reference site (HPRD) in April 2000.

In April of 2000 we collected bullfrog tadpoles from a contaminated site and reference site in close proximity to each other. Perchlorate contamination, thought to have resulted from the on-site manufacturing of solid propellant rocket motors and rocket demilitarization related to the Intermediate-range Nuclear Forces (INF) treaty, has been detected at several sites within LHAAP. One of the most contaminated sites is a water treatment holding pond (site HOLP). The HOLP site directly receives groundwater discharge from a water treatment plant that removes volatile organic solvents from contaminated groundwater but does not remove perchlorate. Perchlorate levels at HOLP have reached as high as 30 ppm since TIEHH first began monitoring perchlorate at this site (Smith et al., 2001) in 1999. In April 2000, perchlorate levels of 1899 Πg perchlorate/L were detected (Table 3). These levels are within a range of sub lethal perchlorate concentrations that completely inhibit metamorphosis in *X. laevis* during continuous exposure through larval life (Goleman et al., 2002a). We did not detect perchlorate in water samples at the HPRD site, which served as a reference site.

The physical characteristics of the HOLP and HPRD sites during collections trips in 2001 are shown in Tables 3 and 4. There was a similar range in water temperature, salinity, and dissolved oxygen in HOLP and HPRD, although the HOLP site tended to be considerably more alkaline.

The HOLP is a semi-permanent pond that receives effluent from the ground water treatment plant. Thus, depth of the pond will vary with effluent discharge. On the other hand, the HPRD is a temporary pond that forms within a depression on the other side of the road from site HOLP.

Our data suggest that the bullfrog tadpoles collected from HOLP and HPRD were the same age. This is supported by whole-body and snout-vent length measurements of these animals (Tables 13-16). Previous work in the *X. laevis* (Goleman et al., 2002a,b) indicates that SVL is not affected by AP concentrations up to 133 ppm. SVL measurements were identical in both groups of tadpoles (Tables 10-12). Life history data collected on natural ranid populations suggest that age and body length are correlated (Sagor et al., 1998, Miaud et al., 1999). In contrast, hindlimb growth was stunted in the HOLP animals (Tables 10-12), averaging 5.2 x shorter in the HOLP animals compared to the HPRD animals. This difference also is reflected in the SVL to HLL ratio, a convenient and non-invasive endpoint for assessing perchlorate exposure. In developing *X. laevis*, more than 90% of the variation in this ratio can be explained by perchlorate concentration (Fig. 4).

Developmental stage in the animals collected at HOLP and HPRD is shown Figs. 2-3. The animals collected from HPRD were in the later stages of prometamorphosis or early metamorphic climax (TK stages XVII-XXXI) (Fig. 3). In contrast, tadpoles collected from the contaminated site had only reached premetamorphosis and early prometamorphosis (TK stages IX-XIII). The quantitative assessment of thyroid gland volume and follicle cell height is presented in Table 13. There were no differences in follicle cell height between the collection sites. In contrast, thyroid gland volume was

2.5-fold larger in tadpoles from the HPRD site compared to tadpoles from the HOLP site.

Analysis of chorus frog tadpoles collected from B25C vicinity in April 2000.

In the process of analyzing thyroid histology from the bullfrog tadpoles collected in April 2000, we determined that the rigorous and labor-intensive quantitative image analysis used on these animals may not be the most efficient method for rapidly screening thyroid histology in dozens of animals. The relatively simple analyses performed in the bullfrog tadpoles took months. Thus, in all subsequent analyses, we employed a rapid histopathological screening methodology designed to efficiently gauge thyroid disruption after perchlorate exposure in mammals (Mann, 2000; Wolf, 2000). Because the thyroid gland is functionally homologous in mammals and amphibians, this method works equally well for assessing thyroid disruption in amphibians. The major utility of this diagnostic tool is that it scores thyroid histology relative to the peculiar etiology that occurs in response to perchlorate exposure: hypertrophy of the follicular epithelium, hyperplasia of the follicular epithelium and colloid depletion. Thus, based on the score for a given animal, one can make a meaningful assessment of whether an individual animal shows signs of perchlorate exposure based on the score for all three characteristics. This turned out to be an ideal method for rapidly screening dozens of animals, most of which showed no signs of thyroid disruption.

B25C-2 and B25C-3 are burned out and partially dismantled cement bunkers with sufficient deep infrastructure remaining to catch significant amounts of rainwater. These buildings were once used to manufacture AP, and perchlorate has been measured in the vicinity of these buildings in the past (Smith et al., 2001). Unfortunately, at the time these animals were collected in April 2000, water levels were not deep enough (a few centimeters deep at most) to collect sufficient water samples for perchlorate analysis. Nonetheless, this site did serve as a breeding area for chorus frogs in the spring of 2000 and we collected developing tadpoles from both sites. In our analysis animals from both sites were stage-matched and we used stage 35-37 tadpoles. As shown in Tables 15 and 17 there was no noticeable colloid depletion, hypertrophy, or hyperplasia in thyroids from tadpoles collected at either site.

Analysis of chorus frog tadpoles collected from B25C vicinity in April 2001.

Buildings B25C-7 and B25C-8 were in the same locality as buildings B25C-2 and B25C-3 that we had previously sampled in April 2000. The previous buildings were not sampled in April 2001 because they had been covered and sealed, preventing access by our research team. However, we did collect dozens of developing chorus frog larvae from B25C-7 and 8. We were also able to collect enough water from each site to perform perchlorate analysis. Analysis of perchlorate revealed that B25C-7 had close to 10 ppm perchlorate while levels in B25C- 8 were nondetectable (*c.f.* Tables 8 and 9). Results of the thyroid histology analysis in stage-matched animals from these two sites are shown in Tables 19 and 21. We immediately noticed distinct hypertrophy and colloid depletion in thyroids from some of the animals collected at B25C-7 (Fig. 5). Semi-quantitative assessment of the thyroids from these animals revealed statistically significant (Table 22) colloid depletion and follicular hypertrophy compared to stage-matched animals from

B25C-8, were perchlorate was not detected. Hyperplasia was not detected in either group.

Analysis of bullfrog tadpoles collected from HPRD in April 2001.

Tables 23 and 24 show developmental and thyroid histopathology data for *R. catesbeiana* tadpoles collected at HPRD in April 2001. There was no evidence of colloid depletion, hypertrophy, or hyperplasia in any of these animals. Perchlorate measured at this site in April 2001 was 126 ppb (Table 4).

Analysis of adult chorus frogs collected at STAR in February and April 2001.

We examined a number of adult chorus frogs that were collected at STAR in February and April 2001. The large majority of these frogs were collected in the leaf litter of the forest floor surrounding the bayou area. Twelve adult male and seven adult female frogs were collected. The gonads appeared normal and oogonia and spermatozoa could easily be recognized based on histological assessment (Tables 25 and 27). There was no evidence of intersex gonads in any of the adults collected from STAR. There was no evidence of significant colloid depletion, follicular hypertrophy or hyperplasia in thyroid tissues from any of these animals (Tables 26 and 28). There was no perchlorate measured in STAR water samples for either February or April 2001 (Table 5).

Analysis of adult green tree frogs collected at HOLP in July 2001.

We examined a number of adult green tree frogs that were collected at HOLP in February and April 2001. Perchlorate levels in HOLP water in July 2001 averaged 146 ± 9 Πg perchlorate/L (Table 3). The frogs were actively calling and breeding and several amplexing pairs were noted but not collected. All of the frogs analyzed were male with the exception of one frog in which the gonads were missing (Table 29). The gonads appeared normal and spermatozoa could easily be recognized based on histological assessment. There was no evidence of significant colloid depletion, follicular hypertrophy or hyperplasia in thyroid tissues from any of these animals (Table 30).

Table 1. Frog Species Collected for ANUR-01-01.

Date	Site	Species	Age	# collected
4/29/00	HOLP	*R. catesbeiana*	larvae	15
"	HPRD	*R. catesbeiana*	larvae	14
	STAR	*R. catesbeiana*	larvae	6
"	STAR	*R. clamitans*	adult	2
"	B25C-2	*P. triseriata*	larvae	8
"	B25C-3	*P. triseriata*	larvae	16
8/25/00	HOLP	*R. clamitans*	larvae	3
"	STAR	*R. catesbeiana*	adult	3
"	STAR	*A. crepitans*	adult	1
"	STAR	*R. c. clamitans*	adult	1
"	HB	*R. catesbeiana*	adult	2
2/24/01	HOLP	*R. catesbeiana*	2 adult, 3 larvae	5
"	HOLP	*R. utricularia*	adult	1
"	HPRD	*R. catesbeiana*	1 adult, 9 larvae	10
"	HPRD II	*R. catesbeiana*	adult	2
"	STAR	*P. triseriata*	adult	12
4/21/01	HOLP	*R. catesbeiana*	larvae	3
"	HPRD	*R. catesbeiana*	larvae	45
"	B25C-7	*P. triseriata*	larvae	100
"	B25C-8	*P. triseriata*	larvae	104
"	STAR	*P. triseriata*	adult	15
7/15/01	HOLP	*H. cinerea*	adult	19
	STAR	*A. crepitans*	adult	14
	STAR	*R. utricularia*	adult	1
7/14/01	STAR	*R. catesbeiana*	larvae	13

Table 2. Specimens Used for Analysis in ANUR-01-01.

Site	Collection date	Species	Number Examined
HOLP	4/29/00	*R. catesbeiana* (larvae)	15
HPRD	4/29/00	*R. catesbeiana* (larvae)	14
B25C-2	4/29/00	*P. triseriata* (larvae)	8
B25C-3	4/29/00	*P. triseriata* (larvae)	13
B25C-7	4/21/01	*P. triseriata* (larvae)	15
B25C-8	4/21/01	*P. triseriata* (larvae)	9
HPRD	4/21/01	*R. catesbeiana* (larvae)	12
STAR	2/24/01	*P. triseriata* (adult)	11
STAR	4/21/01	*P. triseriata* (adult)	8
HOLP	7/14/01	*H. cinerea* (adult)	17

Total = 122.

Table 3. Physical Properties of Site HOLP

Grid reference	UTM: 3615850; GPS: 15S0394869
Source of water	Treatment plant effluent; rainfall
Conductivity	80 ΠS/cm-2052 ΠS/cm
pH	7.77-9.30
Dissolved oxygen	7.34 mg/L
Temperature range	15.8-31.4 °C
Perchlorate analysis	
4/29/00	1899 ± 110 Πg perchlorate/L
8/25/00	6557 ± 10.0 Πg perchlorate/L
2/24/01	106 ± 3.00 Πg perchlorate/L
4/21/01	0.00 ± 0.00 Πg perchlorate/L
7/15/01	142 ± 9.00 Πg perchlorate/L

Table 4. Physical Properties of Site HPRD

Grid reference	UTM: 3615850; GPS: 15S03948260
Source of water	Rainfall
Conductivity	70-560 µS/cm
pH	6.18-7.11
Dissolved oxygen	2.6-7.8 mg/L
Temperature range	16.9-21.1 °C
Perchlorate analysis	
4/29/00	0.00 µg perchlorate/L
2/24/01	0.00 µg perchlorate/L
4/21/01	126 µg perchlorate/L

Table 5. Physical Properties of Site STAR

Grid reference	UTM: 3616958; GPS: 15S0395218
Source of water	Rainfall, lake water
Conductivity	243-560 µS/cm
pH	6.40-6.50
Dissolved oxygen	5.82 mg/L on 7/15/01
Temperature range	23.3-31.5 °C
Perchlorate analysis	
8/25/00	0.00 µg perchlorate/L
2/24/01	0.00 µg perchlorate/L
4/21/01	0.00 µg perchlorate/L
7/15/01	14.5 ± 6.60 µg perchlorate/L

Table 6. Physical Properties of Site B25C-2*

Grid reference	UTM: 3616286; GPS: 15S0392924
Source of water	Rainfall
Conductivity	178.9 µS/cm
pH	8
Dissolved oxygen	7.92 mg/L
Temperature range	NA
Perchlorate analysis	0.00 (trace)

*Measured in the laboratory on October 17, 2000, from water samples collected on August 8, 2000. We were unable to access this site subsequent to this visit because it was boarded up.

Table 7. Physical Properties of Site B25C-3*

Grid reference	UTM: 3616286; GPS: 15S0392924
Source of water	Rainfall
Conductivity	170.9 µS/cm
pH	8
Dissolved oxygen	7.92 mg/L
Temperature range	NA
Perchlorate analysis	0.00

*Measured in the laboratory on October 17, 2000, from water samples collected on August 8, 2000. We were unable to access this site subsequent to this visit because it was boarded up.

Table 8. Physical Properties of Site B25C-7*

Grid reference	UTM: 3616286; GPS: 15S0392924
Source of water	Rainfall
Conductivity	Not measured
pH	Not measured
Dissolved oxygen	Not measured
Temperature range	Not measured
Perchlorate analysis	
4/21/01	9802 Пg perchlorate/L

*Water levels were too low to successfully determine water quality.

Table 9. Physical Properties of Site B25C-8*

Grid reference	UTM: 3616286; GPS: 15S0392924
Source of water	Rainfall
Conductivity	Not measured
pH	Not measured
Dissolved oxygen	Not measured
Temperature range	Not measured
Perchlorate analysis	
4/21/01	0.00 + 0.00 Пg perchlorate/L

*Water levels were too low to successfully determine water quality.

Table 10. Size and Developmental Stage Data for Larval *R. catesbeiana* Collected at HOLP in April 2000.

ID	TK Stage	SVL (mm)	HLL (mm)	Total Length (mm)	Tail Length (mm)	Tail Height (mm)	FLE (Y/N)
HOLP 1	XII	24	6.4	65	41	16	N
HOLP 2	XIV	25	10.5	73	48	18	N
HOLP 3	XIII	26	7.2	68	42	18	N
HOLP 4	XVIII	30	3.9	83	53	19	N
HOLP 5	XI	25	6	70	45	18	N
HOLP 6	X	22	3.1	52	30	16	N
HOLP 7	XIV	28	7.7	75	47	19	N
HOLP 8	IX	24	3	58	34	17	N
HOLP 9		29	4.2	66	37	18	N
HOLP 11	XII	21	3.2	60	39	16	N
HOLP 12	IX	22	2.6	57	35	15	N
HOLP 13	XI	29	5.2	71	42	19	N
HOLP 14	XI	23	4	65	42	18	N
Mean $\pm$ S.E.		25.2 ± 0.83	5.15 ± 0.64	66.4 ± 2.32	41.2 ± 1.74	17.5 ± 0.36	

Table 11. Size and Developmental Stage Data for Larval *R. catesbeiana* Collected at HPRD in April 2000.

ID	TK Stage	SVL (mm)	HLL (mm)	Total Length (mm)	Tail Length (mm)	Tail Height (mm)	FLE (Y/N)
HPRD 1	XX	29	30	76	54	15	Y
HPRD 2	XIX	31	31	80	60	17	N
HPRD 3	XX	29	33	77	54	16	Y
HPRD 4	XXI	25	35	68	42	10	Y
HPRD 5	XX	25	33	74	50	12	Y
HPRD 6	XIX	26	31	72	55	14	N
HPRD 7	XIX	30	32	76	57	10	N
HPRD 8	XX	23	37	74	51	14	Y
HPRD 9	XVII	22	28	74	54	15	N
HPRD10	XVII	30	20	56	32	20	N
HPRD 11	XVII	30	18	52	22	20	N
Mean $\pm$ S.E.		27.3 $\pm$ 0.95	29.8 $\pm$ 1.77	70.8 $\pm$ 2.68	48.3 $\pm$ 3.52	14.8 $\pm$ 1.03	

Table 12. Statistical Analysis of Length Measurements in Bullfrog Tadpoles collected in April 2000.

Location	Perchlorate (ppb)	SVL (mm)	HLL (mm)	HLL/SVL
HPRD	ND	24.3 ± 1.75	26.2 ± 2.36	1.12 ± 0.09
HOLP	1,970	24.9 ± 0.75	5.02 ± 0.60*	0.21 ± 0.02*

ND, Not detectable

Table 13. Thyroid histology in bullfrog tadpoles collected April 2000.

Location	Cell Height (μM)	Thyroid gland volume (mm^3)
HPRD	4.53 ± 0.33 (n=11)	0.035 ± 0.004 (n=10)
HOLP	4.53 ± 0.32 (n=13)	0.014 ± 0.002* (n=13)

HOLP HPRD

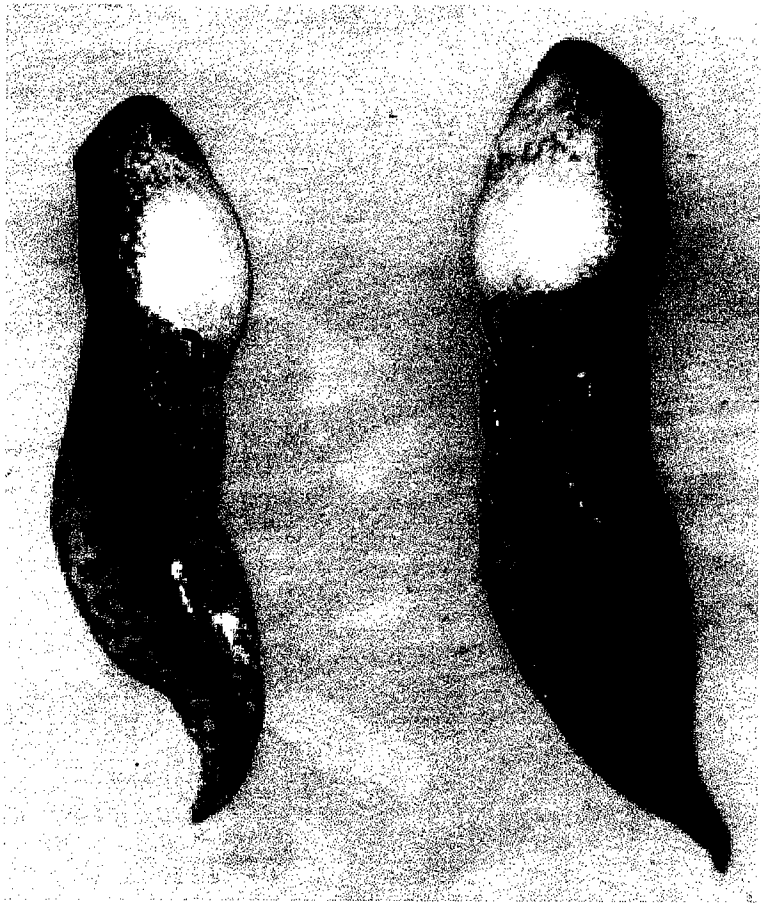

Fig. 1. Comparison of hindlimb lengths (brackets) in *R. catesbeiana* tadpoles collected from HOLP and HPRD. The SVL of these animals are virtually identical, although hindlimb length is 5x less in the animal from HOLP. The HLL/SVL ratio averaged 5 x lower in HOLP animals than HPRD animals (see Table 15).

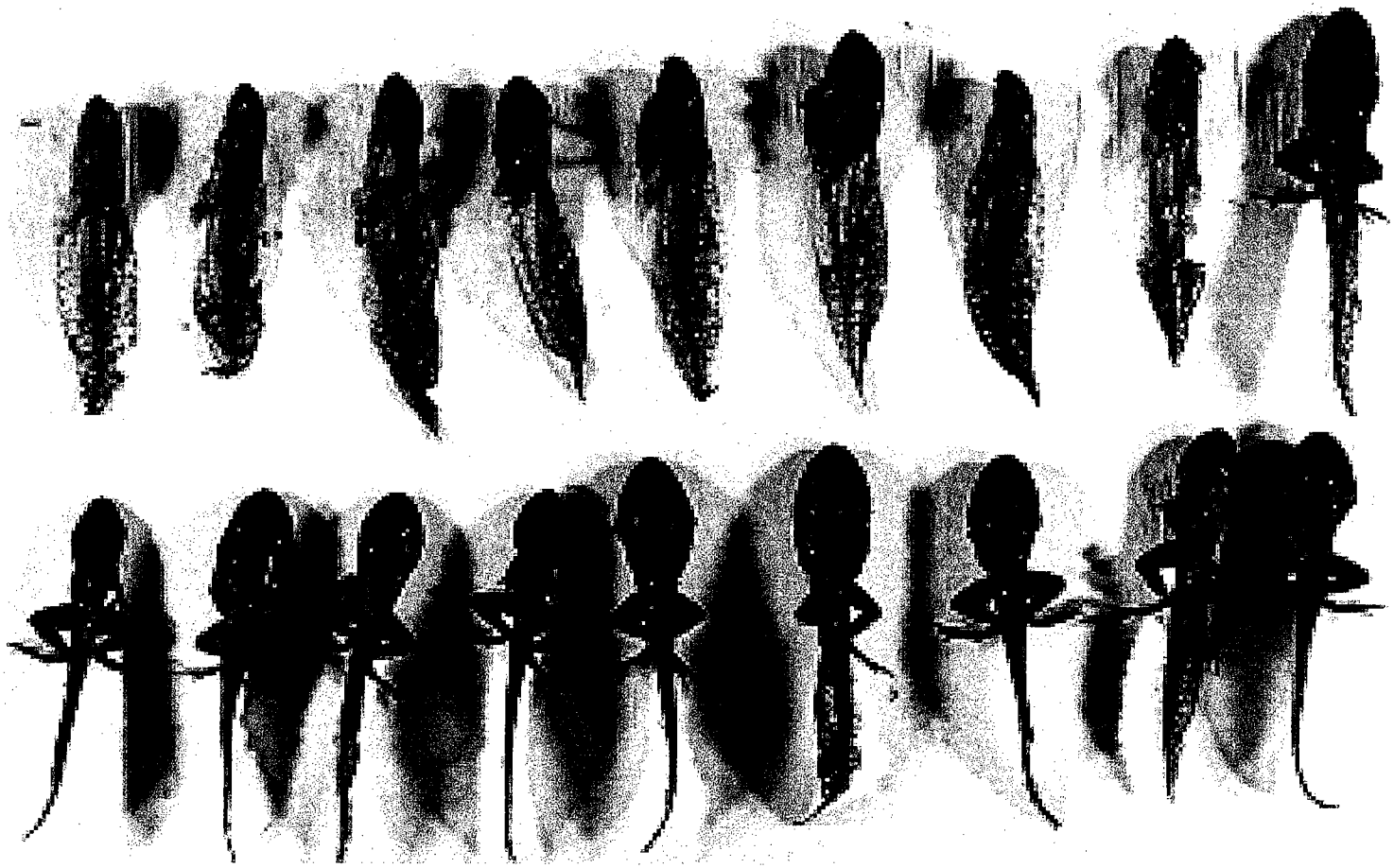

Figure 2. Comparison of *R. catesbeiana* tadpoles collected from HOLP (upper) and HPRD (lower) in April 2000. Only one of the animals collected from HOLP had developed hindlimbs and none of the HOLP animals exhibited forelimb emergence.

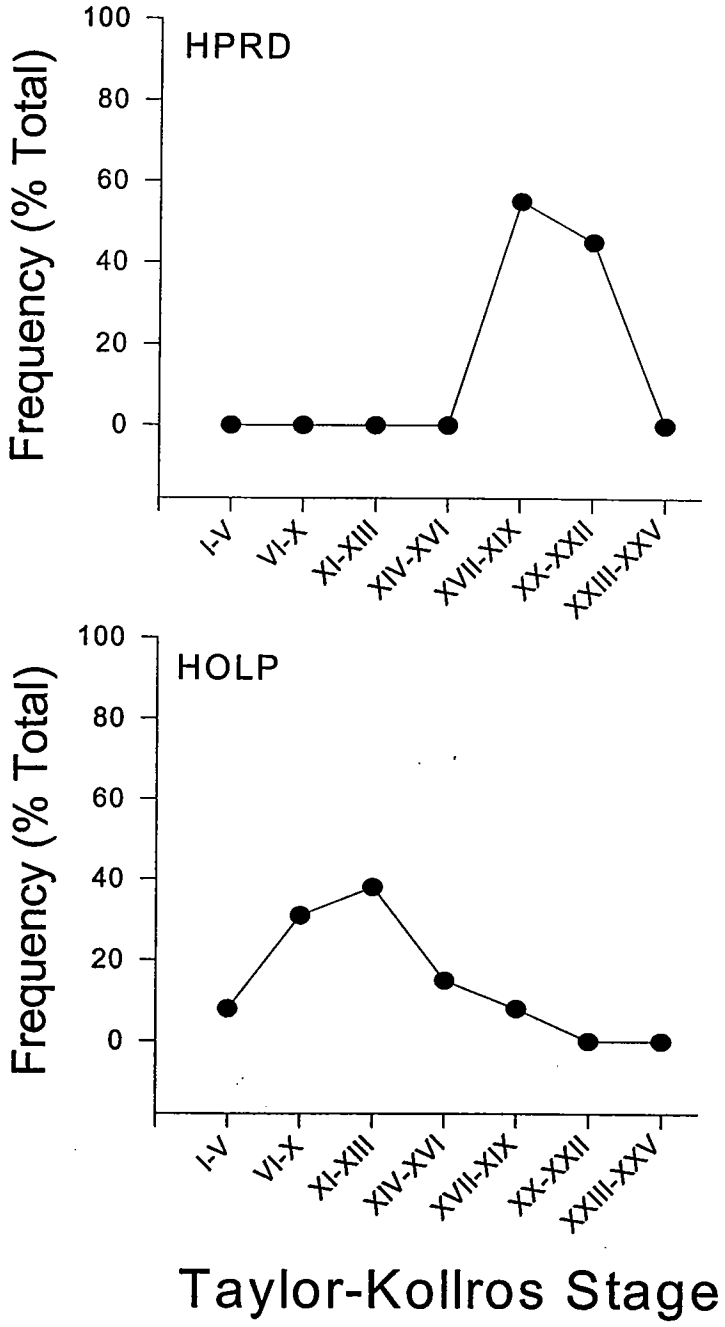

Figure 3. Developmental stage distribution in *R. catesbeiana* tadpoles collected from HOLP and HPRD in April 2000.

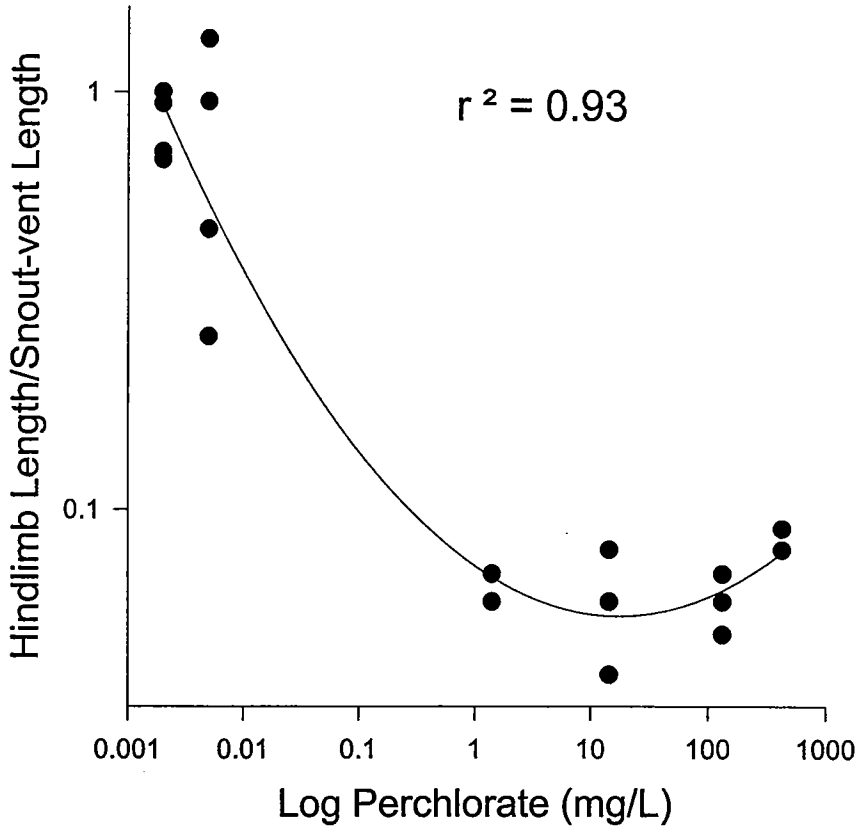

Fig. 4. Perchlorate concentration explains greater than 90% of the variation in hindlimb length/snout-vent length ratio in developing *X. laevis*. Data are regraphed from Goleman et al., 2002a

Table 14. Size and Developmental Stage Data for Larval *P. triseriata* collected at B25C-2 in April 2000.

ID	Gosner Stage	Total length (mm)	SVL (mm)	Tail length (mm)	HLL (mm)	Weight (g)	FLE (Y/N)
B25C-2-1	36	32	14.0	20.0	4	0.494	N
B25C-2-2	36	31	13.5	18.0	4	0.485	N
B25C-2-3	37	28	14.0	16.5	5	0.483	N
B25C-2-4	35	29	12.0	19.0	3	0.381	N
B25C-2-5	37	30	14.0	17.5	5	0.472	N
B25C-2-6	36	31	13.0	21.0	4	0.500	N
B25C-2-7	36	30	13.0	18.0	4	0.438	N
B25C-2-8	35	28	12.0	17.0	3	0.349	N
Mean ± S.E.		29.9 ± 0.51	13.2 ± 0.29	18.4 ± 0.54	4.00 ± 0.26	0.45 ± 0.01	

Table 15. Thyroid histopathology data for larval *P. triseriata* collected at B25C-2 in April 2000.

ID	Colloid Depletion	Hypertrophy	Hyperplasia
B25C-2-1	1.02	0.00	0.00
B25C-2-2	0.06	0.11	0.11
B25C-2-3	0.07	0.00	0.00
B25C-2-4	0.62	0.12	0.00
B25C-2-5	0.59	0.17	0.00
B25C-2-6	0.40	0.31	0.00
B25C-2-7	0.43	0.00	0.00
B25C-2-8	1.67	0.44	0.00
Mean ± S.E.	0.61 ± 0.19	0.14 ± 0.06	0.01 + 0.01

Table 16. Size and Developmental Stage Data for Larval *P. triseriata* collected at B25C-3 in April 2000.

ID	Gosner Stage	Total length (mm)	SVL (mm)	Tail length (mm)	HLL (mm)	Weight (g)	FLE (Y/N)
B25C-3-1	37	31.5	14.5	22	3.50	0.450	N
B25C-3-2	34	26.0	13.0	17	2.00	0.371	N
B25C-3-3	35	24.0	12.0	15	3.00	0.316	N
B25C-3-4	35	23.0	10.0	16	2.00	0.154	N
B25C-3-5	35	21.5	9.50	14	2.00	0.139	N
B25C-3-6	35	23.0	9.50	15	2.00	0.166	N
B25C-3-7	37	25.0	9.00	17	2.50	0.188	N
B25C-3-8	35	23.0	8.00	15	1.50	0.153	N
B25C-3-9	36	22.0	9.00	15	2.50	0.183	N
B25C-3-11	36	25.0	10.5	17	3.00	0.210	N
B25C-3-13	36	22.5	9.00	15	1.50	0.133	N
B25C-3-14	36	24.0	9.00	17	2.50	0.169	N
B25C-3-16	35	23.5	9.50	16	2.00	0.151	N
Mean $\pm$ S.E.		24.2 $\pm$ 0.70	10.2 $\pm$ 0.51	16.2 $\pm$ 0.55	3.30 $\pm$ 0.16	0.21 $\pm$ 0.02	

Table 17. Thyroid Histopathology Data for Larval *P. triseriata* Collected at B25C-2 in April 2000.

ID	Colloid Depletion	Hypertrophy	Hyperplasia
B25C-3-1	0.00	0.00	0.00
B25C-3-2	0.10	0.00	0.00
B25C-3-3	0.00	0.04	0.00
B25C-3-4	0.00	0.00	0.00
B25C-3-5	0.00	0.00	0.00
B25C-3-6	0.00	0.00	0.07
B25C-3-7	0.67	0.00	0.00
B25C-3-8	1.00	0.00	0.00
B25C-3-9	0.00	0.00	0.00
B25C-3-11	1.69	0.23	0.00
B25C-3-13	0.42	0.00	0.00
B25C-3-14	0.17	0.00	0.00
B25C-3-16	0.22	0.00	0.00
Mean $\pm$ S.E.	0.45 $\pm$ 0.18	0.02 $\pm$ 0.02	0.01 + 0.01

Table 18. Size and Developmental Stage Data for Larval *P. triseriata* collected at B25C-7 in April 2001.

ID	Gosner Stage	Total length (mm)	SVL (mm)	Weight (g)	Tail length (mm)	Tail height (mm)	HLL (mm)	FLE (Y/N)
B25C-7-7	34	24	10	0.19	16	7	2.0	N
B25C-7-9	33	25	10	0.19	15	5	1.5	N
B25C-7-10	34	22	10	0.12	15	6	2.0	N
B25C-7-13	33	23	10	0.17	14	4	1.0	N
B25C-7-14	34	26	11	0.22	17	5	1.5	N
B25C-7-21	34	25	10	0.22	15	7	1.5	N
B25C-7-24	34	27	9	0.14	15	4	1.5	N
B25C-7-27	33	26	10	0.19	17	6	1.5	N
B25C-7-28	34	26	10	0.23	16	7	1.5	N
B25C-7-29	34	24	10	0.17	16	7	1.5	N
B25C-7-44	34	23	10	0.16	14	6	1.5	N
B25C-7-46	34	23	10	0.16	16	6	1.5	N
B25C-7-58	34	22	9	0.15	14	5	1.5	N
B25C-7-59	33	22	9	0.15	14	5	1.5	N
B25C-7-60	33	21	9	0.13	14	6	1.0	N
Mean $\pm$ S.E.		23.9 ± 0.12	9.80 ± 0.14	0.17 ± 0.01	15.2 ± 0.27	5.73 ± 0.26	1.50 ± 0.06	

Table 19. Thyroid Histopathology Data for Larval *P. triseriata* Collected at B25C-7 April 2001.

ID	Colloid Depletion	Hypertrophy	Hyperplasia
B25C-7-7	0.22	0.80	0.00
B25C-7-9	0.33	1.25	0.00
B25C-7-10	0.00	0.11	0.00
B25C-7-13	1.00	0.14	0.00
B25C-7-14	1.69	1.76	0.00
B25C-7-21	0.00	0.61	0.00
B25C-7-24	0.14	0.11	0.00
B25C-7-27	2.00	2.00	0.00
B25C-7-28	0.17	2.00	0.00
B25C-7-29	0.08	0.23	0.00
B25C-7-44	0.00	0.07	0.00
B25C-7-46	1.33	0.17	0.00
B25C-7-58	0.00	0.00	0.00
B25C-7-59	1.83	2.00	0.00
B25C-7-60	1.83	0.56	0.00
Mean $\pm$ S.E.	0.71 $\pm$ 0.21	0.79 $\pm$ 0.20	0.00 + 0.00

Table 20. Size and Developmental Stage Data for Larval *P. triseriata* collected at B25C-8 in April 2001.

ID	Gosner Stage	Total length (mm)	SVL (mm)	Weight (g)	Tail length (mm)	Tail height (mm)	HLL (mm)	FLE (Y/N)
B25C-8-40	34	23	9	0.139	16	5	1.5	N
B25C-8-41	34	24	10	0.180	15	6	1.5	N
B25C-8-58	34	24	9	0.167	16	6	1.5	N
B25C-8-66	33	23	8	0.131	16	6	1.5	N
B25C-8-67	34	22	9	0.134	14	5	1.5	N
B25C-8-68	34	22	9	0.124	13	5	1.5	N
B25C-8-69	34	21	8.5	0.122	14	5	1.0	N
B25C-8-70	33	22	8	0.118	14	6	1.0	N
B25C-8-74	33	20	8	0.112	13	5	1.0	N
Mean ± S.E.		22.3 ± 0.44	8.72 ± 0.22	0.13 ± 0.01	14.6 ± 0.41	5.40 ± 0.17	1.30 ± 0.08	

Table 21. Thyroid Histopathology Data for Larval *P. triseriata* Collected at B25C-8 April 2001.

ID	Colloid Depletion	Hypertrophy	Hyperplasia
B25C-8-40	0.00	0.00	0.08
B25C-8-41	0.00	0.00	0.00
B25C-8-58	0.00	0.00	0.00
B25C-8-66	0.00	0.00	0.00
B25C-8-67	0.00	0.00	0.00
B25C-8-68	0.00	0.00	0.08
B25C-8-69	0.17	0.00	0.00
B25C-8-70	0.00	0.00	0.00
B25C-8-74	0.00	0.00	0.00
Mean ± S.E.	0.02 ± 0.02	0.00 ± 0.00	0.02 ± 0.12

Table 22. Thyroid Histopathology in Chorus Frog Tadpoles Collected April 2001.

Location	Perchlorate (ppb)	Gosner Stage	Colloid Depletion	Hypertrophy
B25 C-8	ND	33-34	0.02 ± 0.02 (n=8)	0.00 ± 0.00 (n=8)
B25 C-7	9,802	33-34	0.71 ± 0.2* (n=15)	0.79 ± 0.20* (n=15)

ND, not detectable.

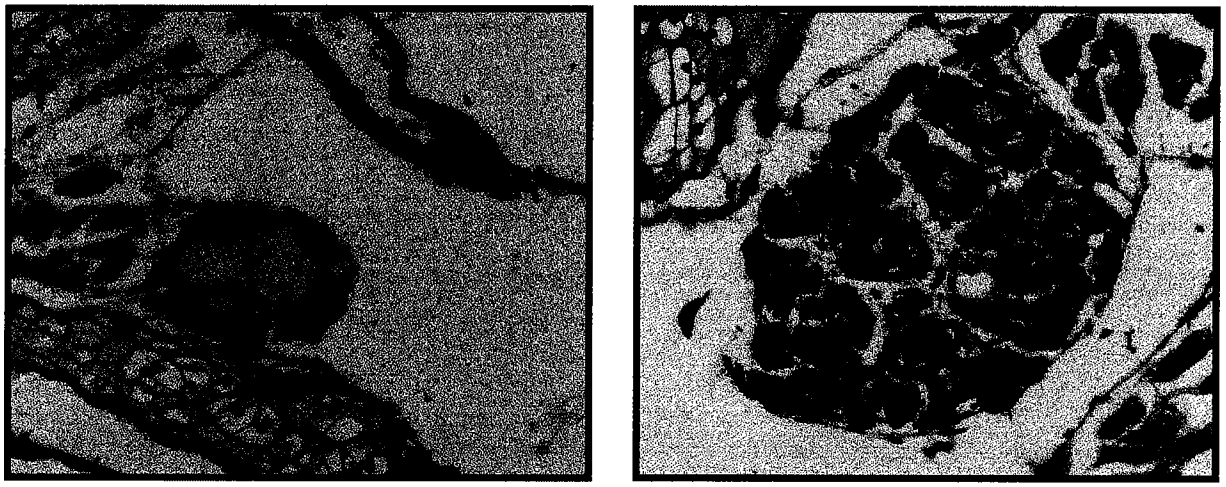

Figure 5. Thyroid disruption in perchlorate-exposed tadpoles. Gosner stage 33-34 chorus frog tadpoles collected from a reference (left) and an AP-contaminated (right) site. Perchlorate levels at the exposed site were 9.8 ppm. Sections were taken from the left gland of stage-matched animals. Note the increased cell height and lack of colloid in the perchlorate-exposed thyroid. These sections were photographed at the same magnification.

Table 23. Size and Developmental Stage Data for Larval *R. catesbeiana* Collected at HPRD in April 2001.

ID	TK Stage	Total length (mm)	SVL (mm)	Tail length (mm)	Tail height (mm)	HLL (mm)	FLE (Y/N)
HPRD 50	XV	ND[b]	29	ND[c]	16	19	N
HPRD 51	XVI	72	27	50	16	18	N
HPRD 55	XIV	76	29	45	16	12	N
HPRD 57	XIV	76	24	51	16	16	N
HPRD 59	XIV	75	28	52	16	15	N
HPRD 63	XV	76	28	52	14	25	N
HPRD 64	XV	80	28	55	16	23	N
HPRD 65	XV	73	27	50	16	22	N
HPRD 66	XIV	74	28	46	16	21	N
HPRD 67	XV	74	30	47	14	32	N
HPRD 68	XIV	ND[b]	27	ND[b]	15	21	N
HPRD 69	XV	73	27	50	18	25	N
Mean ± S.E.		74.9 ± 0.72	27.7 ± 0.43	49.8 ± 0.96	15.8 ± 0.30	20.8 ± 1.53	

[b]ND, tail tip missing; [c]ND, half of tail missing;

Table 24. Thyroid Histopathology Data for Larval *R. catesbeiana* collected at HPRD in April 2001.

ID	Colloid Depletion	Hypertrophy	Hyperplasia
HPRD 50	0.24	0.00	0.00
HPRD 51	0.00	0.00	0.00
HPRD 55	0.07	0.00	0.00
HPRD 57	0.14	0.00	0.00
HPRD 59	0.00	0.00	0.00
HPRD 63	0.13	0.13	0.00
HPRD 64	0.00	0.12	0.00
HPRD 65	0.10	0.14	0.00
HPRD 66	0.00	0.13	0.00
HPRD 67	0.22	0.19	0.00
HPRD 68	0.04	0.14	0.00
HPRD 69	0.00	0.06	0.00
Mean ± S.E.	0.08 ± 0.02	0.08 ± 0.02	0.00 ± 0.00

Table 25. Sex and size data for Adult *P. triseriata* Collected at STAR in February 2001.

ID	Weight (g)	SVL	Sex	Gonadal Histology[a]
STAR 13	0.56	17	F	Stage I-II oocytes
STAR 14	0.62	19	F	Stage I-III oocytes
STAR 15	0.66	16	F	Stage I-II oocytes
STAR 16	0.76	17	M	Spermatozoa present
STAR 17	0.59	17	F	Stage I-III oocytes
STAR 19	0.52	19	M	Spermatozoa present
STAR 20	0.28	14	M	Spermatozoa present
STAR 21	0.43	18	M	Stage I-II oocytes
STAR 22	0.36	17	M	Spermatozoa present
STAR 23	0.50	18	M	Spermatozoa present
STAR 24	0.78	20	M	Spermatozoa present
Mean ± S.E.	0.55 ± 0.04	17.5 ± 0.49		

[a]Oocyte staging based on Dumont (1978).

Table 26. Thyroid Histopathology Data for Adult *P. triseriata* Collected at STAR in February 2001.

ID	Colloid Depletion	Hypertrophy	Hyperplasia
STAR 13	0.42	0.07	0.00
STAR 14	0.18	0.00	0.00
STAR 15	1.22	0.06	0.00
STAR 16	0.00	0.00	0.00
STAR 17	0.36	0.05	0.00
STAR 19	0.23	0.00	0.00
STAR 20	0.47	0.08	0.00
STAR 21	0.80	0.00	0.00
STAR 22	0.98	0.07	0.00
STAR 23	0.89	0.00	0.00
STAR 24	1.00	0.00	0.00
Mean $\pm$ S.E.	0.59 $\pm$ 0.12	0.03 $\pm$ 0.01	0.00 $\pm$ 0.00

Table 27. Sex and size data for Adult *P. triseriata* Collected at STAR in April 2001.

ID	Weight (g)	SVL	Sex	Gonadal Histology
STAR 26	0.787	19	M	Spermatozoa present
STAR 28	0.834	19	M	Spermatozoa present
STAR 30	0.727	18	M	Spermatozoa present
STAR 32	0.834	19	M	Spermatozoa present
STAR 34	0.805	19	M	Spermatozoa present
STAR 35	0.993	19	F	Stage IV oocytes
STAR 37	1.110	20	F	Stage IV oocytes
STAR 40	0.921	21	F	Stage IV oocytes
Mean ± S.E.	0.87 ± 0.04	19.3 ± 0.31		

Table 28. Thyroid Histopathology Data for Adult *P. triseriata* Collected at STAR in April, 2001.

ID	Colloid Depletion	Hypertrophy	Hyperplasia
STAR 26	0.00	0.03	0.00
STAR 28	0.15	0.00	0.00
STAR 30	0.81	0.00	0.00
STAR 32	0.25	0.00	0.00
STAR 34	0.00	0.06	0.00
STAR 35	0.00	0.00	0.00
STAR 37	1.00	0.00	0.00
STAR 40	0.68	0.00	0.00
Mean ± S.E.	0.36 ± 0.14	0.01 ± 0.01	0.00 ± 0.00

Table 29. Sex and size data for Adult *H. cinerea* Collected at HOLP in July 2001.

ID	Weight (g)	SVL	Sex	Gonadal Histology
HOLP 29	6.76	47	M	Spermatozoa present
HOLP 30	6.69	50	M	Spermatozoa present
HOLP 32	6.56	51	M	Spermatozoa present
HOLP 33	8.96	51	M	Spermatozoa present
HOLP 34	8.96	54	M	Spermatozoa present
HOLP 35	8.21	50	M	Spermatozoa present
HOLP 36	7.51	49	?	Gonad not found
HOLP 37	6.22	49	M	Spermatozoa present
HOLP 38	7.54	51	M	Spermatozoa present
HOLP 39	8.05	50	M	Spermatozoa present
HOLP 40	7.13	51	M	Spermatozoa present
HOLP 41	6.87	50	M	Spermatozoa present
HOLP 42	5.98	49	M	Spermatozoa present
HOLP 43	6.83	48	M	Spermatozoa present
HOLP 44	7.65	52	M	Spermatozoa present
HOLP 45	6.81	51	M	Spermatozoa present
HOLP 47	5.31	47	M	Spermatozoa present
Mean $\pm$ S.E.	7.13 $\pm$ 0.24	50.0 $\pm$ 0.42		

Table 30. Thyroid Histopathology Data for Adult *H. cinerea* Collected at HOLP in July 2001.

ID	Colloid Depletion	Hypertrophy	Hyperplasia
HOLP 29	0.24	0.00	0.00
HOLP 30	1.10	0.05	0.00
HOLP 32	0.18	0.00	0.00
HOLP 33	0.73	0.00	0.00
HOLP 34	0.00	0.00	0.00
HOLP 35	0.62	0.06	0.00
HOLP 36	1.58	0.34	0.00
HOLP 37	1.56	0.19	0.00
HOLP 38	1.11	0.07	0.00
HOLP 39	0.72	0.29	0.00
HOLP 40	0.49	0.02	0.00
HOLP 41	0.30	0.00	0.00
HOLP 42	0.06	0.05	0.00
HOLP 43	0.14	0.00	0.00
HOLP 44	0.08	0.00	0.00
HOLP 45	0.35	0.10	0.00
HOLP 47	0.13	0.00	0.00
Mean $\pm$ S.E.	0.55 ± 0.12	0.07 ± 0.03	0.00 ± 0.00

19. DISCUSSION

In theory there are several factors that can determine the extent to which developing frogs can be affected by perchlorate exposure. The first is the variability when perchlorate is present within surface water. This can be affected by several factors including leaching of perchlorate from soil, movement of perchlorate from ground water into surface water, and runoff of perchlorate from surface sediments. Data that have been collected from LHAAP (Tables 3-9) as well as other sites in Texas suggest that the presence of perchlorate can vary significantly from month to month. Thus it is likely that developing frogs will be exposed to intermittent periods of perchlorate. The fact that the effects of perchlorate on metamorphosis are largely reversible (Goleman et al., 2002b) suggest that developing frogs that are exposed intermittently over the course of a long larval period are at less risk for prolonged exposure than frogs that are exposed continuously through a short larval period. For example, bullfrogs frequently over winter as larvae. This long larval period may allow them nonexposure recovery periods. In contrast, rapidly developing species, such as chorus frogs, may not have an opportunity for recovery if exposed for the relatively short length (generally around 2 months) of their larval period. In the present study we found no evidence of thyroid disruption in any of the adult frogs collected from LHAAP, even though high concentrations of perchlorate have been recorded at one of these sites, HOLP, in the past. There may be several explanations for this, but the most parsimonious explanation is that as adults these animals spent less time in the water, thereby minimizing their exposure to perchlorate. We never encountered green tree frogs at HOLP except when they were actively breeding. The fact that exposure to the HOLP only occurred during the few weeks when these animals were breeding may have also reduced their risk of exposure to perchlorate. Similarly, the adult chorus frogs collected at STAR were principally found in the leaf litter of the forest floor surrounding the bayou. Even though there was only one incidence of perchlorate detection, the fact that the adults only returned to the water to breed may have reduced their incidence of exposure.

In contrast, we found evidence for thyroid disruption in chorus frogs inhabiting a perchlorate-manufacturing site at LHAAP and evidence consistent with perchlorate exposure at HOLP. Semi-quantitative histopathological assessment of thyroids from the chorus frogs inhabiting B25C-7 revealed dramatic evidence for thyroid disruption, with hypertrophied follicle cells and virtually no colloid. This dramatic appearance was not observed in animals from any other site. The fact that perchlorate in the water at this site was close to 10 ppm suggests strongly that the histopathological changes in the thyroids of these animals were caused by perchlorate exposure. These same types of histological changes have been observed in *X. laevis* exposed during development (increase follicle cell height, Goleman et al., 2002b) and rats (Siglin et al., 2000). The combination of increased thyroid follicle cell height and colloid depletion are identical to what was observed by Siglin et al. (2000) in rats after a 90 d exposure to perchlorate administered via the drinking water. In order to get sufficient numbers of animals for analysis, we collected the majority of the tadpoles at this site. However, 10 ppm is well above perchlorate concentrations that completely inhibit metamorphosis in *X. laevis* (Goleman et al., 2002a). Thus it is likely that these animals would not have recovered from perchlorate inhibition of thyroid function, and most likely would not have completed

metamorphosis, as there was no obvious drainage from the contaminated site.

We also observed evidence consistent with perchlorate inhibition of thyroid function in bullfrog tadpoles inhabiting HOLP. These animals possessed a 5-fold smaller hindlimb to snout-vent length ratio than size-matched animals from a reference site, HPRD. The animals from the contaminated site had not progressed developmentally to the same stage as animals from the reference site, even though animals from both sites were the same size and, presumably, the same age. The animals from the contaminated site (HOLP) failed to show the increase in thyroid gland size that occurs in late developmental stage animals (such as those collected from HPRD), evidenced by the smaller thyroid gland volume in HOLP tadpoles compared to HPRD tadpoles. This raises an interesting point, as one would expect an increase in thyroid gland volume after chronic perchlorate exposure, with hypertrophy of the gland as seen in the animals collected from B25C-7 (see Figure 5). However, perchlorate levels at B25C-7 were roughly 10 times those at HOLP. Moreover, we have no idea if the animals at HOLP were continuously exposed to perchlorate levels above 1 ppm for their entire developmental period, which is generally 1.5-2 yrs, considerably longer than the chorus frog tadpoles collected at B25C. Our data suggest that animals exposed at HOLP showed retarded development of thyroid-sensitive features of development such as hindlimb growth but that circulating TSH levels were not sufficiently elevated to cause the dramatic hypertrophy seen in B25C-7 animals. Our data do suggest though that developing tadpoles from natural populations exposed to greater than 1 ppm perchlorate show signs of thyroid gland disruption.

20.0 STUDY RECORDS AND ARCHIVE:

Study Records will be maintained at The Institute of Environmental and Human Health (TIEHH) Archive for a minimum of one year after study completion date.

21.0 REFERENCES:

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22.0 APPENDICES:

Study Protocol
Changes to Study Documentation
Change in Study Director
Change in Study No. 1

A STUDY PROTOCOL

ENTITLED

RESPONSE OF NATIVE ADULT AND LARVAL ANURANS IN THEIR NATURAL ENVIRONMENT TO AMMONIUM PERCHLORATE CONTAMINATION: ASSESSMENT OF REPRODUCTIVE AND THYROID ENDPOINTS.

STUDY/PROTOCOL NUMBER: ANUR-01-01

SPONSOR: United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

TESTING FACILITY

Name/Address: Department of Biological Sciences –**AP**
Texas Tech University
Box 4-3131
Lubbock, Texas 79409-3131

Test Facility Management: Dr. James A. Carr

Study Director: Lina J. Urquidi

PROPOSED EXPERIMENTAL START DATE: JUNE 29, 2001

1. **DESCRIPTIVE STUDY TITLE:**
Response of native adult and larval anurans in their natural environment to ammonium perchlorate contamination: assessment of reproductive and thyroid endpoints.

2. **STUDY NUMBER:** ANUR-01-01

3. **SPONSOR:** United States Air Force United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

4. **TESTING FACILITY NAME & ADDRESS:**
Department of Biological Sciences –AP
Texas Tech University
Box 4-3131
Lubbock, TX 79409-3131

5. **PROPOSED EXPERIMENTAL START & TERMINATION DATES:**
Start Date: June 29, 2001
Termination Date: September 30, 2001

6. **KEY PERSONNEL:**
James A. Carr, Testing Facility Manager
Lina J. Urquidi, Study Director
Todd Anderson, Analytical Chemist
Ryan Bounds, Quality Assurance Officer
Ron Kendall, Principal Investigator

7. **DATED SIGNATURES:**

Lina J. Urquidi 6/28/01 — Ms. Lina J. Urquidi, Study Director

7/01/01 — Dr. James Carr, Testing Facility Management

7/3/01 — Mr. Ryan Bounds, Quality Assurance Officer

7-3-01 — Dr. Todd Anderson, Analytical Chemist

7-18-01 — Dr. Ron Kendall, Principal Investigator

5-Aug-01 — Dr. Lou Chiodo, Asst. Director for Science

8. **REGULATORY COMPLIANCE STATEMENT**

Quality Control and Quality Assurance

This study will be conducted in accordance with established Quality Assurance program guidelines and in compliance, where appropriate and possible, with Good Laboratory Practice Standards (40 CFR Part 160, August 17, 1989).

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Dr. James A. Carr
Department of Biological Sciences
Texas Tech University
Box 4-3131
Lubbock, Texas 79409

9. **STUDY OBJECTIVES / PURPOSE:**

To assess the response of the reproductive and thyroid axes in adult and larval anurans from natural populations inhabiting reference sites or sites with known ammonium perchlorate contamination at Longhorn Army Ammunition Plant (LHAAP).

10. TEST MATERIALS:

Test Chemical name: Ammonium Perchlorate
CAS number: 7790-98-9
Characterization: found to be stable in reverse osmosis water for 109 days
Source: contaminated natural surface waters

11. JUSTIFICATION OF TEST SYSTEM

Perchlorate occurs in ground and surface waters in 44 states in the USA, principally as a result of AP discharge from rocket fuel manufacturing facilities or from the demilitarization of missiles (Urbansky, 1998). AP is highly water-soluble and, because reduction of the central chlorine atom occurs very slowly, AP can persist in the environment for decades (Urbansky, 1998).

Ionic perchlorate competitively inhibits thyroidal iodide uptake in mammals (Wolff, 1998) and also disrupts normal thyroid accumulation of iodide in nonmammals including amphibians (Miranda et al., 1996) and lampreys (Manzon and Youson, 1997). The loss of negative feedback due to decreased serum thyroxine (T_4) results in elevated blood thyroid stimulating hormone (TSH) levels and, subsequently, an increase in the height of thyroid follicular epithelial cells (Norris, 1997). Sustained exposure to perchlorate leads to hypertrophy and hyperplasia of follicular cells, resulting in an increased thyroid weight (Siglin et al., 2000).

Although perchlorate salts, principally $NaClO_4$ and $KClO_4$, have been used for years to prevent amphibian metamorphosis, the concentrations used to block metamorphosis are generally greater (250 to 1000 mg/L) than concentrations of perchlorate reported in contaminated surface and ground waters. In a recent study of surface waters and sediments at Longhorn Army Ammunition Plant (LHAAP) in East Texas, perchlorate levels as high as 31.2 ± 0.21 mg perchlorate/L were reported (Smith et al. 2001). Although the perchlorate levels found in contaminated surface waters at LHAAP and elsewhere are well below those traditionally known to prevent metamorphosis in experimental settings, the effects of environmentally relevant concentrations of AP on adult and larval thyroid function are generally unknown.

The goal of the present study is twofold. The first goal is to determine the incidence of thyroid disruption consistent with iodide deficiency in larval and adult anurans in and around LHAAP. The second goal of this study is to assess indices of reproductive activity and development in larval and adult frogs. The bronze frog, *Rana clamitans clamitans*, and the American bullfrog, *Rana catesbeiana*, along with other incidentally collected species, will be used. Both species occur at sites on LHAAP known to be contaminated with perchlorate. Rather than attempt to make direct comparisons of thyroid histopathology between animals from contaminated and reference sites, we will compare the incidence of thyroid histopathology consistent with iodide-deficient goiter: thyroid follicular hypertrophy, microfollicle formation, and colloid depletion (Siglin et al., 2000). In addition, RIA will be used to determine plasma TH, estradiol, and testosterone concentrations in animals from which blood samples are

collected. TH-dependent indices of development and growth (developmental stage, hind-limb length) will also be recorded for larvae. Reproductive indices to be evaluated include gonadal histopathology (incidence of ovo/testes, % atretic follicles), testicular weight, ovary weight, oviductal diameter, vas deferens diameter and fat body weight.

12. **TEST ANIMALS** (Where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test system):
Species: Bronze frog (*Rana clamitans clamitans*), Bullfrog (*Rana catesbeiana*) and other incidentals, such as the Southern leopard frog (*Rana utriculata*), Northern cricket frog (*Acris crepitans*) and the chorus frog (*Pseudacris triseriata*)
Strain: unknown
Age: larvae and adults
Sex: to be determined
Number: approximately 10 adults of each species, and approximately 50 larvae from each of six sites sampled 4 times each.
Source: six field sites: holding pond, holding pond reference ditch, Star Ranch pond, Harrison Bayou "catfish pond", Harrison Bayou downstream from shooting range, Building 25 C, as well as animals previously collected from these sites.

13. **PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:**
Each field site will be assigned a code name. Each collection site will be referenced by its full name or a 4-letter abbreviation. Identified collection sites are holding pond (HOLP), Harrison Bayou "catfish pond" (HBCP), Harrison Bayou downstream (HBDS), holding pond reference ditch (HPRD), Star Ranch Pond (STAR), and building 25-C (B25C). Specimens from each field site will be numbered with the code name and unique identifying number. The project number will be part of the label on each specimen container.

14. **EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:**
Each selected field site will be sampled as follows: (1) sampling will take place for approximately one-two hours by a minimum of two personnel; this will include dipnetting for tadpoles around the circumference of ponds and on each bank of river areas; (2) each site will be sampled four times: approximately mid-spring (April), early summer (June-early July), late summer (August), and early fall (September) during the field season; (3) all anurans captured will be included in sample, regardless of species, sex, or age.

15. **METHODS:**
Field sites were selected based on their history of perchlorate contamination (Smith et all, in press). At each visit to the field site, various compositional and physical parameters will be measured, including:

1. dissolved oxygen

2. air and water temperature
3. conductivity
4. salinity
5. pH
6. anuran species calling

These measures will provide a habitat description of the field site and give information that will be useful when considering the environmental fate of perchlorate. During each visit, a water sample will be collected from the water body. Waters will be collected by hand in clean 4-L glass bottles (**DBS SOP AQ-3-02**) and will be kept cool (i.e. in boxes and/or in shade) during transport to our laboratory where they will be stored at approximately 4 °C until analyzed for perchlorate (**TIEHH SOP AC-2-11**).

Each field site will be visited 3-4 times. If present, approximately 10-50 tadpoles per site will be collected by dip net, quickly anesthetized in 1% 3-aminobenzoic acid ethyl ester (MS-222; **DBS/TCFWRU SOP AF-3-03**) and then fixed in 10% formalin for subsequent thyroid histopathology. If possible, blood collection will be performed by cardiac puncture after anesthesia but prior to placing the animals in formalin (**DBS SOP AQ-3-01**). Adult frogs will be caught by hand and blood samples collected within 5 min of capture by cardiac puncture (**DBS SOP AQ-3-01**). Only adult bullfrogs and bronze frogs will be used for blood collection, as the large size of these animals permits removal of sufficient amounts of blood for analysis without detrimentally affecting the animal's health. The animals will then be returned to their capture site. If available, approximately 6-10 adult animals from each site will be used for assessment of gonadal and thyroid histopathology. After blood collection, these animals will be euthanized in MS-222 and preserved in formalin for gonadal histopathology. All specimen containers will be labeled. Labels will include the project number, appropriate identification number, date of collection, collection site, and collector's initials.

16. ENDPOINT ANALYSIS.

Snout-vent length, developmental stage, total length and body weight will be recorded. If possible, tetraiodothyronine (T_4) will be determined in plasma and extracted from whole larvae as described in **DBS SOPs IN-2-01** and **IN-2-05**. Radioimmunoassays for whole body T_4 and plasma T_4 will be performed according to **DBS SOP IN-2-04** using commercially available antisera and radioisotopes (Goleman and Carr, submitted). Gonads of all fixed animals will be examined by visual inspection to determine sex (as in **DBS SOP IN-2-03**, SOP in preparation). Thyroid glands will be prepared using routine histological methods (**DBS/TCFWRU SOPs IN-1-05, IN-1-06, IN-4-06, IN-4-07, DBS SOPs IN-1-02, IN-1-01, IN-1-04**). Sections will be stained with hematoxylin and eosin (**DBS SOPs IN-1-04**). Thyroid gland activity will be assessed qualitatively for evidence of goiter caused by iodide depletion (enlarged columnar epithelium, depleted colloid, presence of microfollicles [see Siglin et al., 2000; SOP in preparation]). Percent

incidence of goiter will be determined for each site. Percent incidence of intersex gonads will be determined by histological analysis. Developmental state of oogonia and spermatogonia will be determined histologically in adult females and males if sufficient tissue is available.

17. PROPOSED STATISTICAL METHODS

Differences in tail height, tail length, snout-vent length, body weight, thyroid gland epithelial cell height, thyroid gland volume, plasma thyroid hormone levels will be tested independently by one-way ANOVA followed by the Tukey-Kramer multiple comparisons test. Incidence of thyroid gland histopathological characteristics and incidence of gonadal abnormalities will be correlated with attributes of collection site by multivariate regression analysis.

18. REPORT CONTENT/RECORDS TO BE MAINTAINED:

Records to be maintained include field site identification and description, animal identification number (including field site code name and unique number), date of collection, malformity observations, and sex of the collected specimen, mean thyroid follicle cell height, thyroid gland volume, gonadal abnormalities, and plasma TH and reproductive steroid levels.

Report content will include presentation of data, interpretation, and discussion of the following endpoints:

Field study methods
Summary of field sites and number and type of collected specimens
Rate of observed malformities
Analysis of thyroid and reproductive indices
Analysis of TH-dependent aspects of development (larvae only)
Discussion of the relevance of findings
List of all SOPs used

19. RECORDS TO BE MAINTAINED / LOCATION:

The final report will be delivered to the Sponsor on or before November 15, 2001. Copies of all data, documentation, records, protocol information, as well as the specimens shall be sent to the Sponsor, or designated delivery point upon request (within six months of study completion). All data, the protocol and a copy of the final report shall be archived by the testing facility.

20. QUALITY ASSURANCE:

The Quality Assurance Unit will inspect the study at intervals to insure the integrity of the study. Written records will be maintained indicating but not limited to the following: date of inspection, study inspected, phase inspected, person conducting the inspection, findings and problems, recommended and taken action, and any scheduled reinspections.

Any problems likely to effect study integrity shall be brought to the immediate attention of the Study Director. The Quality Assurance Unit will periodically submit written status reports on the study to management and the Study Director.

21. PROTOCOL CHANGES / REVISIONS:

All changes and/or revisions to the protocol, and the reasons therefore, shall be documented, signed and dated by the Study Director and maintained with the protocol and the Quality Assurance Unit.

22. REFERENCES:

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TEXAS TECH
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March 7, 2002

Ron Kendall, Ph.D.
Professor and Director
The Institute of Environmental and Human Health
Texas Tech University
Lubbock, TX 79416

Dear Ron,

Effective August 1, 2001, Ms. Wanda L. Goleman was designated as the study director for project T9700.2, experiment ANUR-01-01.

Sincerely,

James A. Carr, Ph.D.
Associate Professor

Dept. of Biological Sciences (DBS)
Box 43131
Lubbock, TX 79409-3131
(806) 742-2715

Form No. 014 Rev 3. 06/00
Project No.: T9700.2
*Change No: 1
Page: 1 of 2

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: ____ Amendment __X_ Deviation ____ Addendums

Document Reference Information

Check One: __X_ Protocol ____ SOP ____ Other _____

Title: Response of Native Adult and Larval Anurans in Their Natural Environment to Ammonium Perchlorate Contamination: Assessment of Reproductive and Thyroid Endpoints.

Dated: 06/08/01

Document # (if appropriate): T9700.2, ANUR-01-01

Page #(s): 6

Section #: 16

Text to reference: Section 16. ENDPOINT ANALYSIS. Snout-vent length, developmental stage, total length and body weight will be recorded. If possible, tetraiodothyronine (T_4) will be determined in plasma and extracted from whole larvae as described in DBS SOPs IN-2-01 and IN-2-05. Radioimmunoassays for whole body T_4 and plasma T_4 will be performed according to DBS SOP IN-2-04 using commercially available antisera and radioisotopes (Goleman and Carr, submitted). Gonads of all fixed animals will be examined by visual inspection to determine sex (as in DBS SOP IN-2-03, SOP in preparation). Thyroid glands will be prepared using routine histological methods (DBS/TCFWRU SOPs IN-1-05, IN-1-06, IN-4-06, IN-4-07, DBS SOPs IN-1-02, IN-1-01, IN-1-04). Sections will be stained with hematoxylin and eosin (DBS SOPs IN-1-04). Thyroid gland activity will be assessed qualitatively for evidence of goiter caused by iodide depletion (enlarged columnar epithelium, depleted colloid, presence of microfollicles [see Siglin et al., 2000; SOP in preparation]). Percent incidence of goiter will be determined for each site. Percent incidence of intersex gonads will be determined by histological analysis. Developmental state of oogonia and spermatogonia will be determined histologically in adult females and males if sufficient tissue is available.

Change in Document: Section 16. ENDPOINT ANALYSIS. Snout-vent length, developmental stage, total length and body weight will be recorded. If possible, tetraiodothyronine (T_4) will be determined in plasma and extracted from whole larvae as described in DBS SOPs IN-2-01 and IN-2-05. Radioimmunoassays for whole body T_4 and plasma T_4 will be performed according to DBS SOP IN-2-04 using commercially available antisera and radioisotopes (Goleman and Carr, submitted). Gonads of all fixed animals will be examined by visual inspection to determine sex (as in DBS SOP IN-2-03, SOP in preparation). Thyroid glands will be prepared using routine histological methods

* Sequentially numbered in order of the date that the change is effective

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Change In Study Documentation Form

(DBS/TCFWRU SOPs IN-1-05, IN-1-06, IN-4-06, IN-4-07, DBS SOPs IN-1-02, IN-1-01, IN-1-04). Sections will be stained with hematoxylin and eosin (DBS SOPs IN-1-04). Thyroid gland activity will be assessed qualitatively for evidence of goiter caused by iodide depletion (enlarged columnar epithelium, depleted colloid, presence of microfollicles (DBS SOP IN-2-08). Percent incidence of goiter will be determined for each site. Percent incidence of intersex gonads will be determined by histological analysis. Developmental state of oogonia and spermatogonia will be determined histologically in adult females and males if sufficient tissue is available.

Justification and Impact on Study: Section 16. DBS SOP IN-2-08 was finalized on 09/24/01.

Submitted by: Signature: Wanda L. Goleman Date: 2/22/02

Authorized by: Study Director: Wanda L. Goleman Date: 2/22/02

Received by: Quality Assurance Unit: Brian Bidwell Date: 2/22/02

* Sequentially numbered in order of the date that the change is effective

A FINAL REPORT ENTITLED:

THE EFFECTS OF CONTAMINATED AND REFERENCE SURFACE WATERS ON METAMORPHOSIS IN *XENOPUS LAEVIS* USING A MODIFIED US EPA ENDOCRINE DISRUPTOR SCREENING AND TESTING ADVISORY COMMITTEE (EDSTAC, USEPA, 1998)- TIER 1 TAIL RESORPTION ASSAY

STUDY NUMBER:	XEN-01-02
SPONSOR:	Strategic Environmental Research And Development Program (SERDP) 1155 Herndon Parkway, Suite 900 Herndon, Virginia 20170
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
TESTING FACILITY:	Department of Biological Sciences –**AP** Texas Tech University Box 4-3131 Lubbock, Texas 79409-3131
TEST SITE:	Department of Biological Sciences –**AP** Texas Tech University Box 4-3131 Lubbock, Texas 79409-3131
ANALYTICAL TEST SITE:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Center Box 41163 Lubbock, Texas 79409-1163
RESEARCH INITIATION:	March 21, 2001
RESEARCH COMPLETION:	February 18, 2002

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List of Tables and Figures

GOOD LABORATORIES PRACTICES STATEMENT

Project XEN-01-02, entitled "The Effects of Contaminated and Reference Surface Waters on Metamorphosis in *Xenopus laevis* Using a Modified US EPA Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC, USEPA, 1998)- Tier 1 Tail Resorption Assay", was performed whenever possible in the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Noted exceptions are as follows:

None.

Submitted By:

James A. Carr, Ph.D

March 28, 2002
Date

QUALITY ASSURANCE STATEMENT

This study was conducted under the Institute of Environmental and Human Health's Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Any changes in protocol and SOPs were documented in writing and signed by the study director.

The Quality Assurance Officer verbally notified the Study Director of all findings at the time of the inspection. Written audit reports were also submitted to the Study Director and Test Facility Management. Audits were performed for the following phases of the project:

Auditable Research Phase / Activity	Audit Dates		Date written report submitted to Study Director	Date written report submitted to Management
	Start	End		
Mixing of Test Solutions	08/28/01	08/28/01	08/29/01	
Test Material Application	08/29/01	08/29/01	09/04/01	
Final Report	03/04/02	03/20/02		

Submitted By:

Ryan Bounds
Quality Assurance Department

Date 3/28/02

1.0 DESCRIPTIVE STUDY TITLE:
The effects of contaminated and reference surface waters on metamorphosis in *Xenopus laevis* using a modified US EPA Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC, USEPA, 1998)- tier 1 tail resorption assay.

2.0 STUDY NUMBER: XEN-01-02

3.0 SPONSOR:
Strategic Environmental Research
And Development Program (SERDP)
1155 Herndon Parkway, Suite 900
Herndon, Virginia 20170

4.0 TESTING FACILITY NAME AND ADDRESS:
Department of Biological Sciences –**AP**
Texas Tech University
Box 4-3131
Lubbock, Texas 79409-3131

5.0 EXPERIMENTAL START & TERMINATION DATES:
Start: March 21, 2001
Completion: February 18, 2002

6.0 KEY PERSONNEL:
James A. Carr, Testing Facility Manager
Wanda L. Goleman, Study Director
Todd Anderson, Analytical Chemist
Ryan Bounds, Quality Assurance Manager
Ron Kendall, Principal Investigator

7.0 STUDY OBJECTIVES / PURPOSE:
To apply a modified EDSTAC – tier 1 tail resorption test in *Xenopus laevis* to examine the effects of 21-d exposure to ammonium perchlorate (AP) in laboratory preparations and contaminated and reference surface waters collected at Longhorn Army Ammunition Plant (LHAAP) on thyroid gland function beginning with Nieuwkoop and Faber (NF, 1967) stage 55 larvae.

8.0 STUDY SUMMARY:
Twenty *Xenopus* larvae (NF stages 55-66, Nieuwkoop and Faber, 1967) were exposed to one of two laboratory prepared concentrations based on high and low concentrations reported in April 1999 for effluent from the burning ground No. 3 ground water treatment plant (GWTP) located at the Longhorn Army Ammunition plant (LHAAP) in Karnack, TX (AP 14040 ppb or 38 ppb) in FETAX medium or FETAX medium alone. Each treatment was performed in triplicate, with the study repeated once. Surface waters were collected (SOP AQ-3-02) from 2 contaminated and 2 reference sites

located at LHAAP 5 times over 16 months. A modified EDSTAC assay using NF stage 55 – 60 larvae per site/collection was performed with the collected surface waters.

Tail length, tail height, hindlimb length, snout-vent length and NF stage were recorded on Day 0 and at the end of each experiment. Ammonium perchlorate significantly inhibited tail resorption after 45 d of exposure to a concentration of 14040 ppm. Although there was no significant difference in tail length of larvae exposed to natural surface waters, the perchlorate concentrations detected in waters collected for these assays were lower than those previously reported. Our findings suggest that environmentally relevant concentrations of ammonium perchlorate may pose a threat to normal development and growth in natural amphibian populations.

9.0 TEST MATERIALS:

Test Chemical name: Ammonium Perchlorate
CAS number: 7790-98-9
Characterization: 99.999% pure, found to be stable in reverse osmosis water for 109 days.
Source: Aldrich Chemical Company

Test Chemical name: Contaminated and reference surface waters
CAS number: not applicable
Characterization: Field-collected surface waters
Source: LHAAP

Reference Chemical name: deionized water
CAS number: not applicable
Characterization: FETAX (Frog Embryo Teratogenesis Assay- *Xenopus*) medium, a mixture of reagent grade salts, prepared in 100% ultrapure water. The quality of the water was confirmed by analytical tests.
Source: Steam plant condensate water that has been run through reverse osmosis and a de-ionizer to convert it to ultrapure water and contains reagent grade salts in the following concentrations (Sunderman et al., 1991): NaCl, 10.7 mM; $NaHCO_3$, 1.14 mM, KCl, 0.4 mM; $CaCl_2$, 0.14 mM; $CaSO_4$, 0.35 mM, $MgSO_4$, 0.62 mM.

10.0 JUSTIFICATION OF TEST SYSTEM:

Perchlorate prevents intake of iodide from water or food and is goitrogenic (thyroid gland inhibitor) in many animals including fishes and amphibians (Miranda et al., 1996; Manzon and Youson, 1997). Because of the important role played by thyroid hormones in animal development and reproduction, disruption of thyroid function is likely to lead to serious impairments in growth, reproductive fitness, and consequently, amphibian and wildlife population stability as well as human health.

Xenopus are a widely used animal model in basic toxicological, developmental, and reproductive research. Also, there is a considerable database already available for this species. They represent a vertebrate class, amphibians, with distinct developmental and physiological adaptations to their aquatic environment. They are also easily and

economically maintained and bred in the laboratory. Therefore, this species is ideally suited to examine the sublethal effects of AP on aquatic fauna.

11.0 TEST ANIMALS (number, weight, source, strain):

Species: South African Clawed Frog, *Xenopus laevis*
Strain: wild type
Age: Larval stages 55 - 66
Number: approximately 1008
Source: Laboratory colony and *Xenopus* Express, Homosassa, FL.

12.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Each beaker was labeled as indicated in section 5.5 of SOP ET-1-01, which includes genus and species name, common name, project name, number, and start date, sex of the individuals (if appropriate), date eggs were laid/hatched (if applicable), date of initial exposure, and the name of the person responsible for animal care.

13.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Approximately 20 *Xenopus* larvae (NF stages 55-66) were exposed to one of two laboratory prepared concentrations of AP (14040 ppb or 38 ppb) in FETAX medium or FETAX medium alone, for 36 or 45 d. Each treatment was performed in triplicate. This portion of the study was repeated due to an apparent error in mixing the low dose solution. Additionally, the first trial failed within 3 days of initialization. This gave approximately 180 larvae per treatment plus 100 control animals, for a study total of 460 larvae. The AP concentration used represented high and low concentrations reported in April 1999 for effluent from the burning ground No. 3 ground water treatment plant (GWTP) located at the Longhorn Army Ammunition plant (LHAAP) in Karnack, TX.

Surface waters were collected (SOP AQ-3-02) from 2 contaminated and 2 reference sites located at LHAAP during April and August of 2000 and February, April, and July of 2001. A 14 d modified assay using 14 NF stage 60 larvae per beaker and 50% surface waters was run with surface waters collected in April 2000. Surface waters were diluted initially due to concern of a possible increase in mortality from the elevated salinity and conductivity. A 21 d study with 20 NF stage 55 larvae per beaker and 100% surface waters was run with the surface waters collected during August 2000. Contaminated sites identified at LHAAP were the INF treaty (holding) pond (HOLP) and Harrison Bayou catfish pond (HBCP). Identified reference collection sites were the holding pond reference ditch (HPRD) and Star Ranch Pond (STAR). Additionally, one assay was run using waters collected in August 2000 from another contaminated and closely matched reference site identified as building 25-C (B25C). However, since there was only one collection possible from B25C, these data were not included in statistical analyses. Surface waters were collected by hand in clean 4-L glass bottles, transported to Texas Tech University, and stored in a walk-in cooler at approximately 4 ºC in our laboratory until use. Twenty *Xenopus* larvae, NF stage 55, were exposed to each field-collected water or FETAX medium, for 14, 21, 36, or 45 d. This gave either 14 or 20 larvae per collection site X 3 or 4 collection sites X 5 collections plus at least 20 control

animals per collection, for a study total of approximately 548 larvae. (Note: February 2001 and July 2001 surface waters exposures were run concurrently with laboratory solution exposures. Therefore, the FETAX control animals were counted in the total for the laboratory solutions exposures.)

14.0 METHODS:

14.1 Test System acquisition, quarantine, acclimation

Six to twelve adult male and female *Xenopus laevis* were obtained from our lab colony for breeding to obtain eggs for each assay. Refer to SOP AF-1-01 for details on routine *Xenopus* husbandry. They were maintained in 45-L glass tanks containing 18 L of ultrapure water for 1-2 days at approximately 22 $\pm$ 2° C on a 12L: 12D light regimen. Male and female *Xenopus* were maintained separately for at least 7 days before breeding. Please refer to SOP AF-1-02 for details on *Xenopus* breeding.

14.2 Collection of Natural Surface Waters

A total of 5 surface water collections were made: April 2000, August 2000, February 2001, April 2001, and July 2001. For each collection at least 8 L of surface waters were collected from each of 2 contaminated (HOLP and HBCP) and 2 reference (HPRD and STAR) sites. An additional collection site (B25C) was identified in August 2000, with a single collection made at that time from this site . Half of the water collected from 1 site, HPRD, was lost in April 2001 as the bottle was broken during transport. In July 2001 one reference site, HPRD, was dry, therefore no water could be collected. All water collections were made by hand using clean 4 L amber glass bottles. Each bottle was labeled immediately after collection with the location, date, and time of collection as well as the initials of the collector. Collections were kept as cool as possible during transport to Texas Tech University. Upon arrival to our laboratory, collections were immediately placed into a walk-in cooler at approximately 4θC for storage until use. Samples of each water were collected into 20 mL scintillation vials and analyzed for perchlorate content.

14.3 Test condition establishment

Larvae from naturally fertilized eggs were used. They were obtained from three – six pairs of adults who had been artificially induced to spawn (SOP AF-1-02) for each assay. Eggs were collected and a representative sample examined under a microscope for viability (SOP ET-1-01). Fertilized eggs were maintained in 8 – 9 L FETAX medium in 21-L glass tanks. Five-day-old tadpoles were transferred to 45-L glass tanks containing 18 L of FETAX medium. Larvae were allowed to develop to NF stage 55. After reaching NF stage 55 larvae were transferred to 2 L glass beakers containing 1 L of test or reference solution for each assay. Each beaker was labeled as indicated in section 5.5 of SOP ET-1-01, which includes genus and species name, common name, project name and number, sex of the individuals (if appropriate), date eggs were laid/hatched (if applicable), date of initial exposure, and the name of the person responsible for animal care. Larvae

were maintained under temperature – and photoperiod – controlled conditions, 22 ± 2θC and 12: 12 light/ dark, respectively.

14.4 Animal Husbandry

Refer to SOP AF-1-01 for details on routine *Xenopus* husbandry. Adult male and female *Xenopus laevis* were obtained from our lab colony. They were acclimated in 45-L glass tanks containing 18 L of ultrapure water for a minimum of 1-2 days at 22 ± 2° C on a 12L: 12D light regimen. Male and female *Xenopus* were maintained separately for 7 d before breeding. Please refer to SOP AF-1-02 for details on *Xenopus* breeding.

14.5 Test Material Application

Premixed laboratory test solutions or surface waters were added to the appropriately labeled glass beakers (see section 14.2). Larvae were maintained in 2 L glass beakers containing 1 L of laboratory test solutions, surface waters, or FETAX medium. Media was changed every 3 d, with minor exceptions, as indicated for exposure of *Xenopus* to test substances (SOP ET-1-01). Medium containing the identical concentration of test substance was added back to each beaker daily as needed to maintain test conditions.

Rates/concentrations:

Premixed Laboratory Solutions
0, 38 ppb, 14040 ppb

Collected Surface Waters
0 – 5459 ppb

Frequency: Constant exposure for 14 to 45 d

Route/Method of Application: Larvae were exposed to perchlorate in the beaker medium. Larvae were maintained in 1 L of the test solutions in 2 L glass beakers maintained at 22° C ± 2° C for 14, 36, or 45 d. All solutions were changed every 3 d, with minor exceptions, as stated in the SOP for *Xenopus* husbandry (SOP AF-1-01). Method of application was immersion. Route of exposure was via dermal, oral, and respiratory exposure as the chemical was in the beaker medium.

Justification for Exposure Route: *Xenopus* are fully aquatic as larvae and as adults.

14.6 Exposure Verification

Samples of all solutions were analyzed for perchlorate content (TIEHH SOP AC-2-11). After each exposure period 5 larvae per test and reference solution were euthanized in MS-222 (SOP AF-3-03), rinsed in distilled water and frozen for perchlorate analysis.

14.7 Food and Water Trace Contamination

The Cooperative Extension Service at the University of Georgia analyzed food (frog brittle) and water samples for trace contamination. Neither water nor frog brittle contained any detectable pesticide residues. Water contained no detectable trace of heavy metals. Powdered frog brittle contained trace amounts of barium (12 ppm), arsenic (0.39 ppm) and selenium (0.45 ppm) while adult frog brittle contained barium (7.6 ppm), arsenic (0.44 ppm) and selenium (1.03 ppm).

14.8 Test System Observation

Prior to placement in test or reference solutions, larvae were staged (Nieuwkoop and Faber, 1967) and snout-vent length, hindlimb length, and tail length and height measured and recorded. Staging and measurements were repeated at the end of exposure for each tadpole/froglet. Mortality (# dead larvae) was noted every day. Dead animals were removed and preserved in 10% neutral-buffered formalin (NBF). Beginning on the day of exposure, % mortality (#dead larvae/#hatched), % exhibiting deformities, % displaying abnormal swimming behavior, and % metamorphosed animals (# showing complete tail resorption) were noted daily. Time to metamorphosis for each froglet was recorded.

Water quality was monitored regularly. Daily temperatures, room and solutions, were recorded daily. Dissolved oxygen was measured every other day and pH, salinity, conductivity, and ammonia were measured weekly.

14.9 Euthanasia and Sample Collection

After each exposure larvae were staged with snout-vent length, hindlimb length, and tail height and length measured. Animals from all treatments were euthanized by immersion in MS-222 (1g/L, SOP AF-3-03). Approximately 15 animals from each treatment were placed in 10% NBF while 5 animals from each treatment were frozen for subsequent determination of perchlorate content.

14.10 Sample Storage

Samples of all solutions used were stored at approximately 4° C prior to analysis. Animals collected for perchlorate analysis were stored at approximately –29° C until analyzed. The remainder of animals were euthanized and stored in 10% NBF.

14.11 Sample Processing and Sample Analysis

Analysis on the initial dosing solutions has been completed (Tables 9 and 25) with some exceptions. Tissue sample analyses have also been completed (Tables 10 and 26), also with some exceptions.

14.12 Statistical Analysis

Stage-dependent difference in tail height and tail length, as well as snout-vent and hindlimb lengths, were tested independently by one-way ANOVA.

15.0 PROTOCOL CHANGES / REVISIONS:

See attached change in study documentation forms.

16.0 RESULTS:

Tail length, stage data, and statistical summaries for premixed laboratory solutions – experiments 1 and 2 are shown in Tables 1 and 2. It is important to note that larvae developed at a slow rate, thus these assays were extended for an additional 24 d and 15 d, respectively.

Mortality and abnormality data for premixed laboratory solutions – experiments 1 and 2 are shown in Tables 3 – 5. No AP related mortality, bent tails (axial skeleton), asymmetrical tails, or edema were observed in this experiment (Table 5). The effects of AP on swimming behavior and metamorphosis are summarized in Table 6. The effects of AP on larval growth for laboratory solutions – experiments 1 and 2 are summarized in Tables 7 and 8, respectively.

A summary of AP analyses of the initial premixed laboratory dosing solutions is shown in Table 9. Due to the low dose values reported for experiment 1, this portion of the study was repeated. Statistical analyses were performed, however, using the nominal value of 38 ppb AP for experiment 1. There is little discrepancy between nominal and actual AP concentrations for experiment 2. Reported AP concentrations at 38 ppb may not accurately reflect actual AP amount as the potential confounding effects of FETAX medium on the measurement of AP at or near the limit of detection has not been taken into account.

Perchlorate analyses of larvae exposed to the premixed laboratory solutions (Table 10) are currently underway and will be appended to this final report at a later date as appropriate.

Tail length and stage data for surface waters are shown in Tables 11 – 15. It is important to note that the assay with the April 2000 surface waters (Table 11), the waters were diluted to 50% of their original concentrations; the assay was performed with NF stage 60 larvae, and ran for 14 d based on the criteria of the US EPA EDSTAC – tier 1 tail resorption assay. Assays using August 2000 and April 2001 collected waters (Tables 12 and 14) were initiated with NF stage 55 larvae and were ran for 21 d. The assays using collected surface waters from February 2001 (Table 13) and July 2001 (Table 15) were also initiated with NF stage 55 larvae but were extended to 45 d and 36 d, respectively, due to the slow developmental rate of larvae. A statistical summary of tail length and stage data is shown in Table 16.

Mortality for surface waters is shown in Tables 17 – 21, with abnormality data summarized in Table 22. No AP related mortality, bent tails (axial skeleton), asymmetrical tails, or edema were observed in these experiments. The effects of AP on swimming behavior and metamorphosis are summarized in Table 23. The effects of AP on larval growth for surface waters are summarized in Table 24.

A summary of AP analyses for collected surface waters dosing solutions

for April and August 2000 and February and July 2001 is shown in Table 25. Values shown for April 2001 are the analyses for the collected surface waters as the values for the initial dosing solutions have not yet been reported. These data will be appended to this final report at a later date as appropriate.

Perchlorate analyses of larvae exposed to surface waters collected in April and August 2000 and April 2001 are summarized in Table 26. Analyses of larvae exposed to surface waters collected in February and July 2001 are currently underway and will be appended to this final report at a later date as appropriate.

Completion of tail resorption is a thyroid-hormone-dependent process that marks the end of metamorphosis in *Xenopus* and other anuran species. Analysis by one-way ANOVA revealed a significant inhibitory effect ($F_{(2,88)} = 82.43$, $p < 0.0001$; $F_{(2,148)} = 4.99$, $p = 0.008$) of 14040 ppb AP on tail resorption in laboratory prepared solutions (Tables 1 and 2), but not in collected surface waters (Table 16). Similar results were observed in tail height (data not shown). There were also significant differences with respect to snout-vent length ($F_{(2,88)} = 17.16$, $p < 0.0001$; $F_{(2,148)} = 6.96$, $p = 0.0013$) in both premixed laboratory solution experiments. Analysis by one-way ANOVA of snout-vent lengths for larvae exposed to the collected surface waters revealed no such effect. A significant difference was also observed for hindlimb length in experiment 1 ($F_{(2,88)} = 56.72$, $p < 0.0001$), but not in experiment 2 or surface waters. Body weight was not included in these analyses because of the significant reduction in body weight that occurs during metamorphosis, confounding the analysis of treatment effects.

Table 1. Tail Length, Stage Data, and Statistical Summary for Laboratory Solutions – Experiment 1.

		Day 0		Day 45	
Treatment[a]	Sample size	Tail length[b] (mm)	NF Stage[b]	Tail Length[c] (mm)	NF Stage[c]
Control	20	27.1 ± 0.83	55.0 ± 0.00	5.23 ± 2.95[d]	65.0 ± 0.70[d]
AP 38 ppb	60	28.1 ± 0.40	55.0 ± 0.00	5.60 ± 1.72[d]	64.8 ± 0.39[d]
AP 14040 ppb	60	28.6 ± 0.39	55.0 ± 0.00	35.21 ± 1.59[e]	57.4 ± 0.44[e]
F value		$F_{(2,137)} = 1.73$		$F_{(2,88)} = 82.43$	$F_{)2,88)} = 85.85$

Values are expressed as mean ± S.E.M.
[a]Nominal concentrations.
[b]Based on the total number of larvae in each treatment at Day 0.
[c]Based on the total number of surviving larvae in each treatment at Day 45.
Column numbers with different superscripts are significantly different based upon one-way ANOVA followed by the Tukey-Kramer multiple comparisons test.

Table 2. Tail Length, Stage Data, and Statistical Summary for Laboratory Solutions – Experiment 2.

		Day 0		Day 36	
Treatment[a]	Sample size	Tail length[b] (mm)	NF Stage[b]	Tail Length[c] (mm)	NF Stage[c]
Control	60	23.7 ± 0.34[d,e]	55.0 ± 0.00	12.41 ± 1.90[d]	62.6 ± 0.56[d]
AP 38 ppb	60	22.6 ± 0.33[d]	55.0 ± 0.00	19.09 ± 2.07[d,e]	60.7 ± 0.63[d,e]
AP 14040 ppb	60	23.9 ± 0.35[e]	55.0 ± 0.00	21.0 ± 2.17[e]	60.6 ± 4.13[e]
F value		$F_{(2,177)} = 4.14$		$F_{(2,148)} = 4.99$	$F_{(2,148)} = 3.85$

Values are expressed as mean ± S.E.M.
[a]Nominal concentrations.
[b]Based on the total number of larvae in each treatment at Day 0.
[c]Based on the total number of surviving larvae in each treatment at Day 36.
Column numbers with different superscripts are significantly different based upon one-way ANOVA followed by the Tukey-Kramer multiple comparisons test.

Table 3. Mortality Data for Laboratory Solutions – Experiment 1.

Treatment[a]	Sample Size	Mean Mortality (%)
Control	20	35.00
AP 38 ppb	60	23.33
AP 14040 ppb	60	45.00

[a]Nominal concentrations. Values are the mean of all animals per treatment.

Table 4. Mortality Data for Laboratory Solutions – Experiment 2.

Treatment[a]	Sample Size	Mean Mortality (%)
Control	60	35.00
AP 38 ppb	60	20.00
AP 14040 ppb	60	16.67

[a]Nominal concentrations. Values are the mean of all animals per treatment.

Table 5. Abnormality Data for Laboratory Solutions – Experiments 1 and 2.

Treatment[a]	Bent tails (%)	Asymmetric tails (%)	Edema (%)
Control	0	0	0
AP 38 ppb	0	0	0
AP 14040 ppb	0	0	0

[a]Nominal concentrations. Values are the mean of all animals per treatment.

Table 6. Behavioral and Metamorphosis Data for Laboratory Solutions – Experiments 1 and 2.

Treatment[a]	Abnormal Swimming (%)	FLE[b] (%)	Tail Resorption (%)
Control	2.50	66.25	42.50
AP 38 ppb	1.67	62.50	37.50
AP 14040 ppb	5.00	34.17	10.00

[a]Nominal concentrations. Values are the mean of all animals per treatment.
[b]FLE (forelimb emergence)

Table 7. Growth Data for Laboratory Solutions – Experiment 1.

Treatment[a]	SVL[b,c] (mm)	Hindlimb Length[c] (mm)
Control	$16.15 \pm 0.39^{d,e}$	19.56 ± 1.35^{d}
AP 38 ppb	$14.56 \pm 0.25^{d,f}$	$16.59 \pm 0.58^{d,e}$
AP 14040 ppb	$17.52 \pm 0.52^{e,g}$	7.51 ± 0.79^{f}
F value	$F_{(2,88)} = 17.16$	$F_{(2,88)} = 56.72$

Data represent mean ± SEM for the surviving larvae at Day 45.
[a]Nominal concentrations.
[b]SVL, snout-vent length.
[c]Measured on the last day of exposure.
Column numbers with different superscripts are significantly different based upon one-way ANOVA followed by the Tukey-Kramer multiple comparisons test.

Table 8. Growth Data for Laboratory Solutions – Experiment 2.

Treatment[a]	SVL[b] (mm)	Hindlimb Length[c] (mm)
Control	14.34 ± 0.20^{d}	14.47 ± 0.82
AP 38 ppb	14.31 ± 0.23^{d}	11.78 ± 0.98
AP 14040 ppb	15.32 ± 0.22^{e}	12.94 ± 0.90
F value	$F_{(2,148)} = 6.96$	$F_{(2,148)} = 2.26$

Data represent mean ± SEM for the surviving larvae at Day 36.
[a]Nominal concentrations.
[b]SVL, snout-vent length.
[c]Measured on the last day of exposure.
Column numbers with different superscripts are significantly different based upon one-way ANOVA followed by the Tukey-Kramer multiple comparisons test.

Table 9. Results of Laboratory Solutions – Experiments 1 and 2 Dosing Solution Analysis.

Nominal AP	Actual AP (ppb)[a]

	Exp. 1-1	Exp. 1-2	Exp. 1-3	Exp. 2-1	Exp. 2-2	Exp. 2-3
0 ppb	0	NA	NA	0	0	0
38 ppb	279	302	298	64	64	65
14040 ppb	9965	9842	9641	13364	12956	13267

[a]Measured in initial source of each replicate.

Table 10. Results of Perchlorate Analysis for Larvae Exposed to Laboratory Solutions – Experiments 1 and 2.

Nominal AP	Actual AP (ppb)[a]					
	Exp. 1-1[b]	Exp. 1-2[b]	Exp. 1-3[b]	Exp. 2-1[b]	Exp. 2-2[b]	Exp. 2-3[b]
0 ppb						
38 ppb						
14040 ppb						

[a]Measured in 5 larvae per replicate.
[b]Values not yet reported. Analysis in progress.

Table 11. Tail Length and Stage Data for April 2000 Surface Waters.

		Day 0		Day 14	
Treatment	Sample size	Tail length (mm)	NF Stage	Tail Length (mm)	NF Stage
Control	74	37.3 ± 0.31	60.0 ± 0.00	0.00 ± 0.00	66.0 ± 0.00
50% HOLP	14	36.0 ± 0.66	60.0 ± 0.00	0.00 ± 0.00	66.0 ± 0.00
50% HPRD	20	37.1 ± 0.45	60.0 ± 0.00	0.00 ± 0.00	66.0 ± 0.00
50% HBCP	20	38.7 ± 0.68	60.0 ± 0.00	0.05 ± 0.03	65.9 ± 0.00
50% STAR	20	37.0 ± 0.65	60.0 ± 0.00	0.00 ± 0.00	66.0 ± 0.00

Values are expressed as mean ± S.E.M.

Table 12. Tail Length and Stage Data for August 2000 Surface Waters.

	Day 0	Day 21

Treatment	Sample size	Tail length (mm)	NF Stage	Tail Length (mm)	NF Stage
Control	40	32.4 ± 0.80	55.0 ± 0.00	2.91 ± 1.54	65.9 ± 0.29
HOLP	20	28.8 ± 0.88	55.0 ± 0.00	3.33 ± 2.46	65.3 ± 0.31
HPRD	20	35.2 ± 0.57	55.0 ± 0.00	2.06 ± 1.32	65.6 ± 0.15
HBCP	20	33.9 ± 0.60	55.0 ± 0.00	2.17 ± 1.65	65.4 ± 0.20
STAR	20	33.4 ± 0.74	55.0 ± 0.00	3.89 ± 1.72	65.2 ± 0.25

Values are expressed as mean ± S.E.M.

Table 13. Tail Length and Stage Data for February 2001 Surface Waters.

		Day 0		Day 45	
Treatment	Sample size	Tail length (mm)	NF Stage	Tail Length (mm)	NF Stage
Control	20	27.1 ± 0.83	55.0 ± 0.00	2.91 ± 1.54	65.0 ± 0.70
HOLP	20	28.5 ± 0.70	55.0 ± 0.00	3.33 ± 2.46	64.4 ± 0.76
HPRD	20	26.9 ± 0.56	55.0 ± 0.00	2.06 ± 1.32	64.7 ± 0.57
HBCP	20	29.5 ± 0.43	55.0 ± 0.00	2.17 ± 1.65	64.4 ± 0.59
STAR	20	28.7 ± 0.63	55.0 ± 0.00	3.89 ± 1.72	63.8 ± 0.80

Values are expressed as mean ± S.E.M.

Table 14. Tail Length and Stage Data for April 2001 Surface Waters.

		Day 0		Day 21	
Treatment	Sample size	Tail length (mm)	NF Stage	Tail Length (mm)	NF Stage
Control	20	35.8 ± 0.95	55.0 ± 0.00	8.61 ± 3.38	63.3 ± 1.04
HOLP	20	35.8 ± 0.84	55.0 ± 0.00	7.47 ± 3.02	64.0 ± 0.81
HPRD	20	34.8 ± 0.77	55.0 ± 0.00	7.33 ± 2.97	64.2 ± 0.75
HBCP	20	34.2 ± 0.69	55.0 ± 0.00	3.56 ± 2.28	64.6 ± 0.80
STAR	20	36.0 ± 0.98	55.0 ± 0.00	7.40 ± 3.13	63.8 ± 0.84

Values are expressed as mean ± S.E.M.

Table 15. Tail Length and Stage Data for July 2001 Surface Waters.

		Day 0		Day 36	
Treatment	Sample size	Tail length (mm)	NF Stage	Tail Length (mm)	NF Stage

Control	60	23.7 ± 0.34	55.0 ± 0.00	12.41 ± 1.90	62.6 ± 0.56
HOLP	20	23.0 ± 0.94	55.0 ± 0.00	12.19 ± 3.35	62.25 ± 1.1
HBCP	20	24.4 ± 0.54	55.0 ± 0.00	11.00 ± 2.89	61.65 ± 1.09
STAR	20	24.4 ± 0.62	55.0 ± 0.00	11.53 ± 3.32	61.53 ± 1.29

Values are expressed as mean ± S.E.M.

Table 16. Statistical Summary of Ammonium Perchlorate Effects on Metamorphosis for Surface Waters.

		Day 0		Last Day of Exposure	
Treatment	Sample size[a]	Tail Length[a] (mm)	NF Stage[a]	Tail Length[b] (mm)	NF Stage[b]
Control	214	31.45 ± 0.45[c]	60 (74)/ 55 (140)	5.10 ± 0.78	64.64 ± 0.22
HOLP	94	30.04 ± 0.62[c,d]	60 (14)/ 55 (80)	6.80 ± 1.43	64.25 ± 0.38
HPRD	80	33.46 ± 0.53[d]	60 (20)/ 55 (60)	4.52 ± 1.24	65.13 ± 0.24
HBCP	100	32.15 ±5.51[c,d]	60 (20)/ 55 (80)	5.22 ± 1.08	64.35 ± 0.33
STAR	100	32.08 ± 5.47[c,d]	60 (20)/ 55 (80)	6.59 ± 1.25	64.20 ± 0.35
F value		$F_{(4,583)} = 3.93$		$F_{(4,523)} = 0.69$	$F_{(4,523)} = 1.32$

Data represent mean ± S.E.M. of all animals per treatment.
[a]Based on the total number of larvae in each treatment at Day 0. The number of larvae placed into each treatment at the indicated NF stage is shown in parentheses.
[b]Based on the total number of surviving larvae in each treatment on the last day of exposure.
Column numbers with different superscripts are significantly different based upon one-way ANOVA followed by the Tukey-Kramer multiple comparisons test.

Table 17. Mortality Data for April 2000 Surface Waters.

Treatment	Sample Size	Mean Mortality (%)
Control	74	0.00

HOLP	14	0.00
HPRD	20	0.00
HBCP	20	0.00
STAR	20	0.00

Values are the mean of all animals per treatment.

Table 18. Mortality Data for August 2000 Surface Waters.

Treatment	Sample Size	Mean Mortality (%)
Control	40	7.50
HOLP	20	65.00
HPRD	20	0.00
HBCP	20	10.00
STAR	20	5.00

Values are the mean of all animals per treatment.

Table 19. Mortality Data for February 2001 Surface Waters.

Treatment	Sample Size	Mean Mortality (%)
Control	20	35.00
HOLP	20	20.00
HPRD	20	10.00
HBCP	20	10.00
STAR	20	5.00

Values are the mean of all animals per treatment.

Table 20. Mortality Data for April 2001 Surface Waters.

Treatment	Sample Size	Mean Mortality (%)
Control	20	10.00

HOLP	20	5.00
HPRD	20	5.00
HBCP	20	15.00
STAR	20	5.00

Values are the mean of all animals per treatment.

Table 21. Mortality Data for July 2001 Surface Waters.

Treatment	Sample Size	Mean Mortality (%)
Control	60	11.67
HOLP	20	20.00
HBCP	20	0.00
STAR	20	25.00

Values are the mean of all animals per treatment.

Table 22. Abnormality Data for Surface Waters.

Treatment	Bent tails (%)	Asymmetric tails (%)	Edema (%)
Control	0	0	0
HOLP	0	0	0
HPRD	0	0	0
HBCP	0	0	0
STAR	0	0	0

Values are the mean of all animals per treatment.

Table 23. Behavioral and Metamorphosis Data for Surface Waters.

Treatment	Abnormal Swimming (%)	FLE[a] (%)	Tail Resorption (%)
Control	0.93	82.71	69.16

HOLP	2.13	79.79	52.13
HPRD	0.00	95.00	71.25
HBCP	1.00	87.00	62.00
STAR	0.93	83.00	61.00

Values are the mean of all animals per treatment.
[a]FLE (forelimb emergence)

Table 24. Growth Data for Surface Waters Collections.

Treatment	SVL[a,b] (mm)	Hindlimb Length[b] (mm)
Control	15.23 ± 0.12	18.54 ± 0.38
HOLP	15.49 ± 0.21	17.72 ± 0.63
HPRD	15.58 ± 0.21	19.20 ± 0.44
HBCP	15.56 ± 0.19	18.67 ± 0.58
STAR	15.83 ± 0.20	18.07 ± 0.58
F value	$F_{(4,546)} = 1.96$	$F_{(4,523)} = 0.92$

Data represent mean ± SEM of all animals per treatment.
[a]SVL, snout-vent length.
[b]Measured on the last day of exposure.
No significant differences were noted between treatments.

Table 25. Results of Surface Waters Dosing Solution Analysis.

Source	Actual AP (ppb)[a]				
Collection Date	April 2000	August 2000	February 2001	April 2001[b]	July 2001

Control	0	0	0		0
HOLP	1134	5459	86	0	0
HPRD	0	0	0	126	NA
HBCP	0	0	0	14	0
STAR	0	0	0	45	0

[a]Measured in initial dosing solutions.
[b]Measured in collected surface waters. Initial dosing values not yet reported. Analysis in progress.

Table 26. Results of Perchlorate Analysis for Larvae Exposed to Surface Waters.

Source	Actual AP (ppb)[a]				
Collection Date	April 2000	August 2000	February 2001[b]	April 2001	July 2001[a]
Control	0	0		3581	
HOLP	0	1028		2535	
HPRD	0	0		0	NA
HBCP	0	0		2568	
STAR	0	0		2740	

[a]Measured in 5 larvae per treatment.
[b]Values not yet reported. Analysis in progress.

17.0 DISCUSSION

The US EPA EDSTAC – tier I tail resorption assay examines the ability of a chemical to reduce or inhibit thyroid function after hormone synthesis has began. However, we have found this assay lacking the sensitivity to detect the more subtle effects of perchlorate on thyroid hormone (TH) synthesis. Thyroid hormone synthesis is initiated at approximately NF stage 54, the onset of prometamorphosis, and is accumulated in the plasma (Shi, 2000). Perchlorate has been used as a goitrogen for many years to inhibit thyroid activity in clinical and experimental settings. Perchlorate ions block thyroidal iodide uptake by competitively inhibiting the Na^+/I^- symporter (NIS) in thyroid follicle cells, as well as inducing a discharge of pooled intrathyroidal iodide (Wolff, 1998). In some mammalian species perchlorate can accumulate in the thyroid at levels similar to those observed for iodide (Chow et al., 1969; Goldman and Stanbury, 1973). The accumulated perchlorate would not, however, be available to inhibit iodide transport. The potential for environmental perchlorate disruption of thyroid status in humans is supported by work on laboratory rodents. Rats exposed to potassium perchlorate in drinking water exhibit enlarged thyroid glands, elevated levels of TSH in the blood and reduced levels of triiodothyronine (T_3) and thyroxine (T_4) (Mannisto et al., 1979).

In addition to its effects in mammals, perchlorate can alter normal development and metamorphosis in amphibians. Goitrogens that inhibit TH biosynthesis prevent spontaneous metamorphosis in amphibians and produce overgrown larvae (Galton, 1988). *Xenopus* larvae exposed to environmentally relevant concentrations of ammonium perchlorate in tank medium also exhibit enlarged thyroid glands and reduced levels of whole-body T_4 (Goleman and Carr, 2002, in press). Anuran metamorphosis can be divided into three stages (Just and Kraus-Just, 1996): 1) premetamorphosis, which includes the larval morphological stages that proceed in the absence of the thyroid gland, therefore presumably little or no TH, 2) prometamorphosis, which includes the developmental stages that require the presence of TH, and 3) metamorphic climax, which includes only the larval stages that require a hypothalamic-dependent surge of TH levels. During prometamorphosis there is a gradual increase in the level of circulating TH. Maximum TH levels occur after forelimb emergence in *Xenopus* (Nieuwkoop and Faber [NF] stage 58) (Nieuwkoop and Faber 1967), and decline again just prior to complete tail resorption (NF stage 66).

Our results indicate that concentrations of perchlorate reported in surface waters of the INF treaty pond containing waste from a water treatment plant at the Longhorn Army Ammunition Plant in Karnack, TX causes direct interference with TH synthesis and secretion in the developing larvae, thereby decreasing the rate of metamorphosis in prometamorphic *Xenopus* tadpoles. While no obvious abnormalities such as bent tails (axial skeleton) or edema were observed, completion of tail resorption was significantly reduced in premixed laboratory solution treatment animals. The slowed, but continued development in treated animals over the length of the assay is probably related not only to the length of time required to discharge iodide from the thyroid, but also the continued renewal

of an iodide source, namely the frog brittle (48 mg/kg) fed to the larvae. Tadpoles received 0.1 gm of frog brittle, which translates into 4.8 Πg of iodide added to the treatment solutions, with each feeding. The primary source of inorganic iodide is dietary. Inorganic iodide is absorbed from the intestine into the blood and accumulated by thyroid follicular cells. Although the effects of perchlorate are mediated by blocking the NIS, it is a reversible, competitive inhibition.

Although a recent study of surface waters and sediments at Longhorn Army Ammunition Plant (LHAAP) in East Texas, found perchlorate levels as high as 31.2 ± 0.21 ppm (Smith et al. 2001), the highest concentration found in the surface waters collected for these assays was 5459 ppb. These assays have shown no significant reduction in tail resorption in *Xenopus* larvae exposed to surface waters collected from LHAAP. One reason for these results may be the amount of iodide available for uptake by the tadpoles. Not only was iodide present in the frog brittle but also in the surface waters collected for these assays (up to 180 ppb). Thus it is likely that the uptake of iodide by the NIS was simply reduced by the presence of perchlorate instead of being blocked completely.

Declining amphibian populations have been reported worldwide. The potential ramifications of thyroid hormone disruption in amphibians are serious, as thyroid hormones are required for normal development and metamorphosis. An increase in the time to complete metamorphosis due to a decreased developmental rate in natural amphibian populations could be devastating to the population, depending on the life history of the species. A species with a short larval period, for instance, developing in ephemeral pools may not be able to complete metamorphosis before the water evaporates. The ability of AP to disrupt thyroid activity in natural amphibian populations and the potential role of this contaminant in declining amphibian populations are important areas for continued research.

18.0 STUDY RECORDS AND ARCHIVE:

Study Records will be maintained at The Institute of Environmental and Human Health (TIEHH) Archive for a minimum of one year after study completion date.

19.0 REFERENCES:

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20.0 APPENDICES:

Study Protocol
Changes to Study Documentation

A STUDY PROTOCOL

ENTITLED

THE EFFECTS OF CONTAMINATED AND REFERENCE SURFACE WATERS ON METAMORPHOSIS IN *XENOPUS LAEVIS* USING A MODIFIED US EPA ENDOCRINE DISRUPTOR SCREENING AND TESTING ADVISORY COMMITTEE (EDSTAC, USEPA, 1998)- TIER 1 TAIL RESORPTION ASSAY

STUDY/PROTOCOL NUMBER: XEN-01-02

SPONSOR: United States Air Force
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TESTING FACILITY

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Test Facility Management: Dr. James A. Carr

Study Director: Wanda L. Goleman

PROPOSED EXPERIMENTAL
START DATE March 21, 2001

1. **DESCRIPTIVE STUDY TITLE:**
The effects of contaminated and reference surface waters on metamorphosis in *Xenopus laevis* using a modified US EPA Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC, USEPA, 1998)- tier 1 tail resorption assay.

2. **STUDY NUMBER:** XEN-01-02

3. **SPONSOR:** United States Air Force United States Air Force
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5. **PROPOSED EXPERIMENTAL START & TERMINATION DATES:**
Start Date: March 21, 2001
Termination Date: September 30, 2001

6. **KEY PERSONNEL:**
James A. Carr, Testing Facility Manager
Wanda L. Goleman, Study Director
Todd Anderson, Analytical Chemist
Ryan Bounds, Quality Assurance Officer
Ron Kendall, Principal Investigator

7. **DATED SIGNATURES:**

Wanda L. Goleman 3/15/01	Ms. Wanda L. Goleman Study Director
3/15/01	Dr. James Carr Testing Facility Management
3/21/01	Mr. Ryan Bounds Quality Assurance Officer
Todd Anderson 3-21-01	Dr. Todd Anderson Analytical Chemist
Ronald J. Kendall 3/21/01	Dr. Ron Kendall Principal Investigator

8. **REGULATORY COMPLIANCE STATEMENT**

Quality Control and Quality Assurance

This study will be conducted in accordance with established Quality Assurance program guidelines and in compliance, where appropriate and possible, with Good Laboratory Practice Standards (40 CFR Part 160, August 17, 1989).

Document Control Statement

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Dr. James A. Carr
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Texas Tech University
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Lubbock, Texas 79409

9. **STUDY OBJECTIVES / PURPOSE:**

To apply a modified EDSTAC Tier 1 tail resorption test in *Xenopus laevis* to examine the effects of 21-d exposure to ammonium perchlorate (AP) in laboratory preparations and contaminated and reference surface waters collected at Longhorn Army Ammunition Plant (LHAAP) on thyroid gland function beginning with Nieuwkoop and Faber (NF, 1967) stage 55 larvae.

10. TEST MATERIALS:

Test Chemical name: Ammonium Perchlorate
CAS number: 7790-98-9
Characterization: 99.999% pure, found to be stable in reverse osmosis water for 109 days.
Source: Aldrich Chemical Company

Test Chemical name: Contaminated and reference surface waters
CAS number: not applicable
Characterization: Field-collected surface waters
Source: LHAAP

Reference Chemical name: deionized water
CAS number: not applicable
Characterization: FETAX (Frog Embryo Teratogenesis Assay- *Xenopus*) medium, a mixture of reagent grade salts, prepared in 100% ultrapure water. The quality of the water will be confirmed by analytical tests.
Source: Steam plant condensate water that has been run through reverse osmosis and a de-ionizer to convert it to ultrapure water and contains reagent grade salts in the following concentrations (Sunderman et al., 1991): NaCl, 10.7 mM; $NaHCO_3$, 1.14 mM, KCl, 0.4 mM; $CaCl_2$, 0.14 mM; $CaSO_4$, 0.35 mM, $MgSO_4$, 0.62 mM.

11. JUSTIFICATION OF TEST SYSTEM

Perchlorate prevents intake of iodide from water or food and is goitrogenic (thyroid gland inhibitor) in many animals including fishes and amphibians (Miranda et al., 1996; Manzon and Youson, 1997). Because of the important role played by thyroid hormones in animal development and reproduction, disruption of thyroid function is likely to lead to serious impairments in growth, reproductive fitness, and consequently, amphibian and wildlife population stability as well as human health.

Xenopus are a widely used animal model in basic toxicological, developmental, and reproductive research. Also, there is a considerable database already available for this species. They represent a vertebrate class, amphibians, with distinct developmental and physiological adaptations to their aquatic environment. They are also easily and economically maintained and bred in the laboratory. Therefore, this species is ideally suited to examine the sublethal effects of AP on aquatic fauna.

12. **TEST ANIMALS** (Where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test system):
Species: South African Clawed Frog, *Xenopus laevis*
Strain: wild type
Age: Larval stages 55 - 66
Number: Approximately 580
Source: Laboratory colony and *Xenopus* Express, Homosassa, FL.

13. **PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:**
Each beaker will be labeled as indicated in section 5.8 of **DBS SOP #AF-1-02**, which includes genus and species name, common name, project name, number, animal receipt date, sex of the individuals (if appropriate), date eggs were laid/hatched (if applicable), and the name of the person responsible for animal care, as well as the date of initial exposure, if applicable.

14. **EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:**
Approximately 20 *Xenopus* larvae (NF stages 55-66, Nieuwkoop and Faber, 1967) will be exposed to one of two laboratory prepared concentrations of AP in FETAX medium or FETAX medium alone, for 21 d. Each treatment will be performed in triplicate. This will give approximately 20 larvae per treatment, for a study total of approximately 180 larvae. The AP concentration to be used represent high and low concentrations reported in April 1999 for effluent from the burning ground No. 3 ground water treatment plant (GWTP) located at the Longhorn Army Ammunition plant (LHAAP) in Karnack, TX.

Surface waters will be collected (**DBS SOP #AQ-3-02**) from 2 contaminated and 2 reference sites located at LHAAP approximately bimonthly, if possible, from February to September 2001. Each collection site will be referenced by its full name or a 4-letter abbreviation. Identified contaminated collection sites are holding pond (HOLP) and Harrison Bayou catfish pond (HBCP). Identified reference collection sites are holding pond reference ditch (HPRD) and Star Ranch Pond (STAR). Waters will be collected by hand in clean 4-L glass bottles and will be kept cool during transport to our laboratory where they will be stored in a walk-in cooler at 4° C. Approximately 20 *Xenopus* larvae (NF stages 55-66, Nieuwkoop and Faber, 1967) will be exposed to each field-collected water or FETAX medium, for 21-d. This will give approximately 20 larvae per collection site X 4 collection sites X 4 months plus 20 control animals per collection, for a study total of approximately 400 larvae.

15. **METHODS:**
Six adult male and female *Xenopus laevis* will be obtained from our lab colony and *Xenopus* Express. Refer to **DBS SOP #AF-1-01** for details on routine *X. laevis* husbandry. They will be maintained in 45-L glass tanks containing 18 L of ultrapure reverse osmosis water for 1-2 days at approximately 20 $\pm$ 2° C on a 12L: 12D light

regimen. Male and female *X. laevis* will be maintained separately for 7 days before breeding. Please refer to **DBS SOP #AF-1-02** for details on *X. laevis* breeding.

15.1 Test System acquisition, quarantine, acclimation

Larvae from naturally fertilized eggs will be used. They will be obtained from three pairs of adults who have been artificially induced to spawn (see *X. laevis* husbandry **DBS SOP #AF-1-02**). These eggs will be collected and a representative sample examined under a microscope for viability (**DBS SOP #ET-1-01**). Fertilized eggs will be maintained in 9 L FETAX medium in 21-L glass tanks. 5-d tadpoles will be transferred to 45-L glass tanks containing 18 L of FETAX medium. Larvae will be allowed to develop to NF stage 55. After reaching NF stage 55 approximately 20 larvae will be transferred to 2000-mL glass beakers containing 1000-mL of test or reference solution. Each beaker will be labeled as indicated in section 5.8 of **DBS SOP #AF-1-02**, which includes genus and species name, common name, project name and number, animal receipt date, sex of the individuals (if appropriate), date eggs were laid/hatched (if applicable), and the name of the person responsible for animal care, as well as the date of initial exposure.

15.2 Test condition establishment

Based on preliminary experiments using NF stages 54 and 55 larvae in FETAX medium, a modified assay based on the EDSTAC tail resorption assay (Fort and Stover 1997) has been developed. After 21 days 79% of NF stage 55 animals had completed tail resorption while only 53% of NF stage 54 animals had completed tail resorption. Thus, we have modified the EDSTAC assay to begin with prometamorphic NF stage 55 larvae and extended the assay to 21 days to allow the less developed larvae adequate time for complete tail resorption.

15.3 Test Material Application

For laboratory preparations, test material will be premixed to appropriate concentrations and added to the appropriately labeled glass beakers (see section 15.1). Larvae will be maintained in 2000-mL glass beakers containing 1000-mL of test solution in FETAX medium or FETAX medium alone.

Field-collected surface waters will be added to the appropriately labeled glass beakers (see section 15.1) allowed to come to room temperature (20 ± 2° C). Larvae will be maintained in 2000-mL glass beakers containing 1000-mL of test solution in FETAX medium or FETAX medium alone.

Medium will be changed as indicated for exposure of *X. laevis* to test substances (**DBS SOP #ET-1-01**). Medium containing the identical concentration of test substance will be added back to each beaker daily as needed to maintain test conditions.

Surface waters will be changed as indicated for exposure of *X. laevis* to test substances (**DBS SOP #ET-1-01**). Water from each respective collection site/time will be added back to the appropriate beaker daily as needed to maintain test conditions.

Rates/concentrations: 0, 38 ppb, 14 040 ppb AP.
Field-collected surface waters will be analyzed for AP content.

Frequency: Constant exposure for 21 d.

Route/Method of Application: Larvae will be exposed to AP in the beaker medium. Larvae will be maintained in 1000-mL of the test solution in 2000-mL glass beakers maintained at 22° C ± 2° C for 21 days. Method of application will be immersion. Route of exposure will be via dermal, oral, and respiratory exposure as the chemical will be in the beaker medium.

Justification for Exposure Route: *X. laevis* are fully aquatic as larvae and as adults.

Exposure Verification: Samples of test and reference solutions will be analyzed for AP content (**TIEHH SOP #AC-2-11**). After the 21-d test period 5 larvae per test and reference solution will be euthanized in MS-222 (**DBS/TCFWRU SOP #AF-3-03**), rinsed in distilled water and frozen for AP analysis.

15.4 Test System Observation

Prior to placement in test or reference solutions, larvae will be staged (Nieuwkoop and Faber, 1967) and tail length and height recorded. Mortality (# dead larvae), percent showing deformities, percent displaying abnormal swimming behavior, percent showing forelimb emergence (FLE, both forelimbs visible) and percent metamorphosed animals (complete tail resorption) will be noted daily throughout the experiment. Dead animals will be removed daily and preserved in 10% neutral-buffered formalin (NBF).

15.5 Animal Sacrifice and Sample Collections

After 21 d of exposure larvae will be staged and tail height and length measured. Animals from each treatment will be euthanized by immersion in 3-aminobenzoic acid ethyl ester (MS-222, 1g/L, **DBS/TCFWRU SOP #AF-3-03**). Approximately 15 animals from each treatment will be placed in 10% NBF while approximately 5 animals from each treatment will be frozen on dry ice for subsequent determination of AP content.

15.6 Endpoint Analysis

Tail length and height will be measured and recorded on Day 0 and Day 21 for subsequent analysis of tail resorption.

16. PROPOSED STATISTICAL METHODS

Stage-dependent difference in tail height and tail length will be tested independently by one-way ANOVA followed by the Tukey-Kramer multiple comparisons test.

17. REPORT CONTENT/RECORDS TO BE MAINTAINED:

Records to be maintained include:

- Room temperature and water temperature, salinity, pH, dissolved oxygen content, and conductivity will be collected.
- Date, time, frequency and amount of feedings per beaker will be recorded. Number of expired larvae removed prior to termination of exposure will be recorded, including time, date, and beaker.

Report content will also include presentation of data, interpretation, and discussion of the following end-points:

- Discussion of the relevance of the findings
- List of all SOPs used.
- List of all personnel.

18. RECORDS TO BE MAINTAINED / LOCATION:

The final report will be delivered to the Sponsor on or before November 15, 2001. A copy of all data, documentation, records, protocol information, as well as the specimens shall be sent to the Sponsor, or designated delivery point upon request (within six months of study completion). All data, the protocol and a copy of the final report shall be archived by the testing facility.

19. QUALITY ASSURANCE:

The Quality Assurance Unit will inspect the study at intervals to insure the integrity of the study. Written records will be maintained indicating but not limited to the following: date of inspection, study inspected, phase inspected, person conducting the inspection, findings and problems, recommended and taken action, and any scheduled reinspections. Any problems likely to effect study integrity shall be brought to the immediate attention of the Study Director. The Quality Assurance Unit will periodically submit written status reports on the study to management and the Study Director.

20. PROTOCOL CHANGES / REVISIONS:

All changes and/or revisions to the protocol, and the reasons therefore, shall be documented, signed and dated by the Study Director and maintained with the protocol and the Quality Assurance Unit.

20. REFERENCES:

Fort D, Stover E. 1997. Development of short-term, whole-embryo assays to evaluate detrimental effects on amphibian limb development and metamorphosis using *Xenopus laevis*. In Dwyer FJ, Doane TR, Hinman ML, eds, *Environmental Toxicology and Risk Assessment: Modeling and Risk Assessment*, Vol 6-ASTM STP 1317. American Society for Testing and Materials. Philadelphia, PA. USA, pp 376-390.

Manzon, R.G. and Youson, J.H. (1997). The effects of exogenous thyroxine (T_4) on triiodothyronine (T_3), in the presence or absence of potassium perchlorate, on the incidence of metamorphosis and on serum T_4 and T_3 concentrations in larval sea lampreys (*Petromyzon marinus* L). Gen. Comp. Endocrinol. 106: 211-220.

Miranda, L.A., Pisano, A. and Casco, V. (1996). Ultrastructural study of thyroid glands of *Bufo arenarum* larvae kept in potassium perchlorate solution. Biocell 20: 147-153.

Nieuwkoop, P.D. and Faber, J. (1967). Normal table of *Xenopus laevis* (Daudin), North Holland, Amsterdam.

Sunderman, F.W., Plowman, M.C. and Hopfer, S.M. (1991). Embryotoxicity and teratogenicity of cadmium chloride in *Xenopus laevis*, assayed by the FETAX procedure. Ann. Clin. Lab. Sci. 21: 381-391.

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Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: ___ Amendment __X_ Deviation ____ Addendums

Document Reference Information

Check One: __X_ Protocol ____ SOP ____ Other _____

Title: The effects of contaminated and reference surface waters on metamorphosis in *Xenopus laevis* using a modified US EPA Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC, USEPA, 1998)- tier 1 tail resorption assay.

Dated: March 21, 2001

Document # (if appropriate): T9700.2; XEN-01-02

Page #(s): 5, 6,7

Section #: 14, 15.2, 15.3, 15.5, 15.6

Text to reference:

Section 14. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:
Approximately 20 *Xenopus larvae* (NF stages 55-66, Nieuwkoop and Faber, 1967) will be exposed to one of two laboratory prepared concentrations of AP in FETAX medium or FETAX medium alone, for 21 d. Each treatment will be performed in triplicate. This will give approximately 20 larvae per treatment, for a study total of approximately 180 larvae. The AP concentration to be used represent high and low concentrations reported in April 1999 for effluent from the burning ground No. 3 ground water treatment plant (GWTP) located at the Longhorn Army Ammunition plant (LHAAP) in Karnack, TX.

Surface waters will be collected (DBS SOP #AQ-3-02) from 2 contaminated and 2 reference sites located at LHAAP approximately bimonthly, if possible, from February to September 2001. Each collection site will be referenced by its full name or a 4-letter abbreviation. Identified contaminated collection sites are holding pond (HOLP) and Harrison Bayou catfish pond (HBCP). Identified reference collection sites are holding pond reference ditch (HPRD) and Star Ranch Pond (STAR). Waters will be collected by hand in clean 4-L glass bottles and will be kept cool during transport to our laboratory where they will be stored in a walk-in cooler at 4° C. Approximately 20 Xenopus larvae (NF stages 55-66, Nieuwkoop and Faber, 1967) will be exposed to each field-collected water or FETAX medium, for 21-d. This will give approximately 20 larvae per collection site X 4 collection sites X 4 months plus 20 control animals per collection, for a study total of approximately 400 larvae.

Section 15.2. Test condition establishment: Based on preliminary experiments using NF stages 54 and 55 larvae in FETAX medium, a modified assay based on the EDSTAC tail resorption assay (Fort and Stover 1997) has been developed. After 21 days 79% of NF stage 55 animals had completed tail resorption while only 53% of NF stage 54 animals had completed tail resorption. Thus, we have modified the EDSTAC assay to begin with

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prometamorphic NF stage 55 larvae and extended the assay to 21 days to allow the less developed larvae adequate time for complete tail resorption.
Section 15.3. Test Material Application: Route/Method of Application: Larvae will be exposed to AP in the beaker medium. Larvae will be maintained in 1000-mL of the test solution in 2000-mL glass beakers maintained at 22° C ± 2° C for 21 days. Method of application will be immersion. Route of exposure will be via dermal, oral, and respiratory exposure as the chemical will be in the beaker medium.
Section 15.5. Animal Sacrifice and Sample Collections: After 21 d of exposure larvae will be staged and tail height and length measured. Animals from each treatment will be euthanized by immersion in 3-aminobenzoic acid ethyl ester (MS-222, 1g/L, **DBS/TCFWRU SOP #AF-3-03**). Approximately 15 animals from each treatment will be placed in 10% NBF while approximately 5 animals from each treatment will be frozen on dry ice for subsequent determination of AP content.
Section 15.6. Endpoint Analysis: Tail length and height will be measured and recorded on Day 0 and Day 21 for subsequent analysis of tail resorption.

Change in Document:
Section 14. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:
Approximately 20 *Xenopus larvae* (NF stages 55-66, Nieuwkoop and Faber, 1967) will be exposed to one of two laboratory prepared concentrations of AP in FETAX medium or FETAX medium alone, for 45 d. Each treatment will be performed in triplicate. This will give approximately 20 larvae per treatment, for a study total of approximately 180 larvae. The AP concentration to be used represent high and low concentrations reported in April 1999 for effluent from the burning ground No. 3 ground water treatment plant (GWTP) located at the Longhorn Army Ammunition plant (LHAAP) in Karnack, TX.

Surface waters will be collected (DBS SOP #AQ-3-02) from 2 contaminated and 2 reference sites located at LHAAP approximately bimonthly, if possible, from February to September 2001. Each collection site will be referenced by its full name or a 4-letter abbreviation. Identified contaminated collection sites are holding pond (HOLP) and Harrison Bayou catfish pond (HBCP). Identified reference collection sites are holding pond reference ditch (HPRD) and Star Ranch Pond (STAR). Waters will be collected by hand in clean 4-L glass bottles and will be kept cool during transport to our laboratory where they will be stored in a walk-in cooler at 4° C. Approximately 20 Xenopus larvae (NF stages 55-66, Nieuwkoop and Faber, 1967) will be exposed to each field-collected water or FETAX medium, for 45 d. This will give approximately 20 larvae per collection site X 4 collection sites X 4 months plus 20 control animals per collection, for a study total of approximately 400 larvae.
Section 15.3. Test Material Application: Route/Method of Application: Larvae will be exposed to AP in the beaker medium. Larvae will be maintained in 1000-mL of the test solution in 2000-mL glass beakers maintained at 22° C ± 2° C for 45 days. Method of

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application will be immersion. Route of exposure will be via dermal, oral, and respiratory exposure as the chemical will be in the beaker medium.

Section 15.5. Animal Sacrifice and Sample Collections: After 45 d of exposure larvae will be staged and tail height and length measured. Animals from each treatment will be euthanized by immersion in 3-aminobenzoic acid ethyl ester (MS-222, 1g/L, **DBS/TCFWRU SOP #AF-3-03**). Approximately 15 animals from each treatment will be placed in 10% NBF while approximately 5 animals from each treatment will be frozen on dry ice for subsequent determination of AP content.

Section 15.6. Endpoint Analysis: Tail length and height will be measured and recorded on Day 0 and Day 45 for subsequent analysis of tail resorption.

Justification and Impact on Study:

Section 14, Section 15.2, Section 15.3, Section 15.5, Section 15.6. The length of exposure was extended due to the slow development of all larvae, including control and reference animals. It was previously established that larvae develop at different rates. Unfortunately the larvae used in the experiment with laboratory prepared solutions and reference and contaminated surface waters collected in February 2001 were not the first of the pool to reach NF stage 55. It was due to the necessity of re-ordering glassware before the experiment could begin. Even with extending this trial to 45 d all control larvae did not reach NF stage 66. Although the final results should not be affected, the usefulness of the modified experiment as a short-term assay, in this particular trial, is vastly decreased.

Submitted by: Signature: Jranda L. Goleman Date: 5/12/01

Authorized by: Study Director: Jranda L. Goleman Date: 5/12/01

Received by: Quality Assurance Unit: [signature] Date: 3/19/02

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Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: X Amendment ____ Deviation ____ Addendums

Document Reference Information

Check One: X Protocol ____ SOP ____ Other ____

Title: The effects of contaminated and reference surface waters on metamorphosis in *Xenopus laevis* using a modified US EPA Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC, USEPA, 1998)- tier 1 tail resorption assay.
Dated: March 21, 2001
Document # (if appropriate): XEN-01-02
Page #(s): 5
Section #: 12, 14

Text to reference: Section 12. TEST ANIMALS (Where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test system): Number: Approximately 580.
Section 14. Experimental Design Including Bias Control: Approximately 20 *Xenopus* larvae (NF stages 55-66, Nieuwkoop and Faber, 1967) will be exposed to one of two laboratory prepared concentrations of AP in FETAX medium or FETAX medium alone, for 21 d. Each treatment will be performed in triplicate. This will give approximately 20 larvae per treatment, for a study total of approximately 180 larvae. The AP concentration to be used represent high and low concentrations reported in April 1999 for effluent from the burning ground No. 3 ground water treatment plant (GWTP) located at the Longhorn Army Ammunition plant (LHAAP) in Karnack, TX.

Surface waters will be collected (DBS SOP #**AQ-3-02**) from 2 contaminated and 2 reference sites located at LHAAP approximately bimonthly, if possible, from February to September 2001. Each collection site will be referenced by its full name or a 4-letter abbreviation. Identified contaminated collection sites are holding pond (HOLP) and Harrison Bayou catfish pond (HBCP). Identified reference collection sites are holding pond reference ditch (HPRD) and Star Ranch Pond (STAR). Waters will be collected by hand in clean 4-L glass bottles and will be kept cool during transport to our laboratory where they will be stored in a walk-in cooler at 4° C. Approximately 20 *Xenopus* larvae (NF stages 55-66, Nieuwkoop and Faber, 1967) will be exposed to each field-collected water or FETAX medium, for 21-d. This will give approximately 20 larvae per collection site X 4 collection sites X 4 months plus 20 control animals per collection, for a study total of approximately 400 larvae.

Change in Document: Section 12. TEST ANIMALS (Where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test

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system): Number: Approximately 960.
Section 14. Experimental Design Including Bias Control:
Approximately 20 *Xenopus* larvae (NF stages 55-66, Nieuwkoop and Faber, 1967) will be exposed to one of two laboratory prepared concentrations of AP in FETAX medium or FETAX medium alone, for 21 d. Each treatment will be performed in triplicate. This will give approximately 20 larvae per treatment, for a study total of approximately 360 larvae. The AP concentration to be used represent high and low concentrations reported in April 1999 for effluent from the burning ground No. 3 ground water treatment plant (GWTP) located at the Longhorn Army Ammunition plant (LHAAP) in Karnack, TX.

Surface waters will be collected (DBS SOP #**AQ-3-02**) from 2 contaminated and 2 reference sites located at LHAAP approximately bimonthly, if possible, from February to September 2001. Each collection site will be referenced by its full name or a 4-letter abbreviation. Identified contaminated collection sites are holding pond (HOLP) and Harrison Bayou catfish pond (HBCP). Identified reference collection sites are holding pond reference ditch (HPRD) and Star Ranch Pond (STAR). Waters will be collected by hand in clean 4-L glass bottles and will be kept cool during transport to our laboratory where they will be stored in a walk-in cooler at 4° C. Approximately 20 *Xenopus* larvae (NF stages 55-66, Nieuwkoop and Faber, 1967) will be exposed to each field-collected water or FETAX medium, for 21-d. Data collected during earlier assays using surface waters collected during April and August 2000 will also be included in the statistical analysis. This will give approximately 20 larvae per collection site X 4 collection sites X 6 months plus 20 control animals per collection, for a study total of approximately 600 larvae.

Justification and Impact on Study: Section 12./ Section 14. (1) There was an error made in mixing the laboratory low concentration of AP. To correct for this error, this study must be repeated which increases the number of animals used. (2) Due to the rapid decrease in ammonium perchlorate contamination at LHAAP from remediation efforts results of earlier modified EDSTAC assays will be included in the final analyses of this study. The sampling locations from these previous studies are identical to those named for this study. The addition of data from studies utilizing the natural waters collected during April and August 2000 will increase the statistical validity by increasing the overall sample size.

Submitted by: Signature: Randall K. Coleman Date: 5/21/01

Authorized by: Study Director: Randall K. Coleman Date: 5/21/01

Received by: Quality Assurance Unit: [signature] Date: 3/19/02

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Document Reference Information

Check One: X Protocol ____ SOP ____ Other ____

Title: The effects of contaminated and reference surface waters on metamorphosis in *Xenopus laevis* using a modified US EPA Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC, USEPA, 1998)- tier 1 tail resorption assay.
Dated: March 21, 2001
Document # (if appropriate): XEN-01-02; Change in Study 1; Change in Study 2
Page #(s): (XEN-01-02) 5; (Change in Study 1) 1; (Change in Study 2) 1
Section #: 12, 14

Text to reference: Section 12. TEST ANIMALS (Where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test system): Number: Approximately 960.
Section 14. Experimental Design Including Bias Control:
Approximately 20 *Xenopus* larvae (NF stages 55-66, Nieuwkoop and Faber, 1967) will be exposed to one of two laboratory prepared concentrations of AP in FETAX medium or FETAX medium alone, for 21 d. Each treatment will be performed in triplicate. This will give approximately 20 larvae per treatment, for a study total of approximately 360 larvae. The AP concentration to be used represent high and low concentrations reported in April 1999 for effluent from the burning ground No. 3 ground water treatment plant (GWTP) located at the Longhorn Army Ammunition plant (LHAAP) in Karnack, TX.

Surface waters will be collected (DBS SOP #**AQ-3-02**) from 2 contaminated and 2 reference sites located at LHAAP approximately bimonthly, if possible, from February to September 2001. Each collection site will be referenced by its full name or a 4-letter abbreviation. Identified contaminated collection sites are holding pond (HOLP) and Harrison Bayou catfish pond (HBCP). Identified reference collection sites are holding pond reference ditch (HPRD) and Star Ranch Pond (STAR). Waters will be collected by hand in clean 4-L glass bottles and will be kept cool during transport to our laboratory where they will be stored in a walk-in cooler at 4° C. Approximately 20 *Xenopus* larvae (NF stages 55-66, Nieuwkoop and Faber, 1967) will be exposed to each field-collected water or FETAX medium, for 21-d. Data collected during earlier assays using surface waters collected during April and August 2000 will also be included in the statistical analysis. This will give approximately 20 larvae per collection site X 4 collection sites X 6 months plus 20 control animals per collection, for a study total of approximately 600 larvae.

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Change in Document: Section 12. TEST ANIMALS (Where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test system): Number: Approximately 880.
Section 14. Experimental Design Including Bias Control:
Surface waters will be collected (DBS SOP #**AQ-3-02**) from 2 contaminated and 2 reference sites located at LHAAP approximately bimonthly, if possible, from February to July 2001. Each collection site will be referenced by its full name or a 4-letter abbreviation. Identified contaminated collection sites are holding pond (HOLP) and Harrison Bayou catfish pond (HBCP). Identified reference collection sites are holding pond reference ditch (HPRD) and Star Ranch Pond (STAR). Waters will be collected by hand in clean 4-L glass bottles and will be kept cool during transport to our laboratory where they will be stored in a walk-in cooler at 4° C. Approximately 20 *Xenopus* larvae (NF stages 55-66, Nieuwkoop and Faber, 1967) will be exposed to each field-collected water or FETAX medium, for 21-d. Data collected during earlier assays using surface waters collected during April and August 2000 will also be included in the statistical analysis. This will give approximately 20 larvae per collection site X 4 collection sites X 6 months plus 20 control animals per collection, for a study total of approximately 520 larvae.

Justification and Impact on Study: Section 12./ Section 14. Due to the rapid decrease in perchlorate contamination at LHAAP from remediation efforts the concentrations of perchlorate have been drastically reduced, rendering further analysis of LHAAP field water by this method inadequate.

Submitted by: Signature: Wanda L. Goleman Date: 8/27/01

Authorized by: Study Director: Wanda L. Goleman Date: 8/27/01

Received by: Quality Assurance Unit: [signature] Date: 3/19/02

* Sequentially numbered in order of the date that the change is effective

Dept. of Biological Sciences (DBS)
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(806) 742-2715

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: X Amendment ___ Deviation ___ Addendums

Document Reference Information

Check One: X Protocol ___ SOP ___ Other ___

Title: The effects of contaminated and reference surface waters on metamorphosis in *Xenopus laevis* using a modified US EPA Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC, USEPA, 1998)- tier 1 tail resorption assay.

Dated: March 21, 2001

Document # (if appropriate): T9700.2; XEN-01-02 protocol; Change in Study No. 1; Change in Study No. 2; Change in Study No. 3

Page #(s): Protocol: 5, 6, 7
Change in Study No. 1: 2
Change in Study No. 2: 1
Change in Study No. 3: 2

Section #: 14, 15.2, 15.3, 15.5, 15.6

Text to reference:

Section 14. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:
Approximately 20 *Xenopus larvae* (NF stages 55-66, Nieuwkoop and Faber, 1967) will be exposed to one of two laboratory prepared concentrations of AP in FETAX medium or FETAX medium alone, for 45 d. Each treatment will be performed in triplicate. This will give approximately 20 larvae per treatment, for a study total of approximately 180 larvae. The AP concentration to be used represent high and low concentrations reported in April 1999 for effluent from the burning ground No. 3 ground water treatment plant (GWTP) located at the Longhorn Army Ammunition plant (LHAAP) in Karnack, TX.

Surface waters will be collected (DBS SOP #**AQ-3-02**) from 2 contaminated and 2 reference sites located at LHAAP approximately bimonthly, if possible, from February to July 2001. Each collection site will be referenced by its full name or a 4-letter abbreviation. Identified contaminated collection sites are holding pond (HOLP) and Harrison Bayou catfish pond (HBCP). Identified reference collection sites are holding pond reference ditch (HPRD) and Star Ranch Pond (STAR). Waters will be collected by hand in clean 4-L glass bottles and will be kept cool during transport to our laboratory where they will be stored in a walk-in cooler at 4° C. Approximately 20 *Xenopus* larvae (NF stages 55-66, Nieuwkoop and Faber, 1967) will be exposed to each field-collected water or FETAX medium, for 21-d. Data collected during earlier assays using surface waters collected during April and August 2000 will also be included in the statistical analysis. This will give approximately 20 larvae per collection site X 4 collection sites X

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6 months plus 20 control animals per collection, for a study total of approximately 520 larvae.

Section 15.3. Test Material Application: Route/Method of Application: Larvae will be exposed to AP in the beaker medium. Larvae will be maintained in 1000-mL of the test solution in 2000-mL glass beakers maintained at 22° C ± 2° C for 45 days. Method of application will be immersion. Route of exposure will be via dermal, oral, and respiratory exposure as the chemical will be in the beaker medium.

Section 15.5. Animal Sacrifice and Sample Collections: After 45 d of exposure larvae will be staged and tail height and length measured. Animals from each treatment will be euthanized by immersion in 3-aminobenzoic acid ethyl ester (MS-222, 1g/L, **DBS/TCFWRU SOP #AF-3-03**). Approximately 15 animals from each treatment will be placed in 10% NBF while approximately 5 animals from each treatment will be frozen on dry ice for subsequent determination of AP content.

Section 15.6. Endpoint Analysis: Tail length and height will be measured and recorded on Day 0 and Day 45 for subsequent analysis of tail resorption.

Change in Document:

Section 14. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:
Approximately 20 *Xenopus larvae* (NF stages 55-66, Nieuwkoop and Faber, 1967) will be exposed to one of two laboratory prepared concentrations of AP in FETAX medium or FETAX medium alone until approximately 80% of control larvae have completed tail resorption, or supply of field-collected surface waters (concurrent study) have been depleted. Each treatment will be performed in triplicate. This will give approximately 20 larvae per treatment, for a study total of approximately 180 larvae. The AP concentration to be used represent high and low concentrations reported in April 1999 for effluent from the burning ground No. 3 ground water treatment plant (GWTP) located at the Longhorn Army Ammunition plant (LHAAP) in Karnack, TX.

Surface waters will be collected (SOP **AQ-3-02**) from 2 contaminated and 2 reference sites located at LHAAP approximately bimonthly, if possible, from February to July 2001. Each collection site will be referenced by its full name or a 4-letter abbreviation. Identified contaminated collection sites are holding pond (HOLP) and Harrison Bayou catfish pond (HBCP). Identified reference collection sites are holding pond reference ditch (HPRD) and Star Ranch Pond (STAR). Waters will be collected by hand in clean 4-L glass bottles and will be kept cool during transport to our laboratory where they will be stored in a walk-in cooler at 4° C. Approximately 20 *Xenopus* larvae (NF stages 55-66, Nieuwkoop and Faber, 1967) will be exposed to each field-collected surface water or FETAX medium until approximately 80% of control larvae have completed tail resorption, or supply of field-collected surface waters have been depleted. Data collected during earlier assays using surface waters collected during April and August 2000 will also be included in the statistical analysis. This will give approximately 20 larvae per

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collection site X 4 collection sites X 6 months plus 20 control animals per collection, for a study total of approximately 520 larvae.

Section 15.3. Test Material Application: Route/Method of Application: Larvae will be exposed to AP in the beaker medium. Larvae will be maintained in 1000-mL of the test solution in 2000-mL glass beakers maintained at 22° C ± 2° C until approximately 80% of control larvae have completed tail resorption, or supply of field-collected surface waters have been depleted. Method of application will be immersion. Route of exposure will be via dermal, oral, and respiratory exposure as the chemical will be in the beaker medium.

Section 15.5. Animal Sacrifice and Sample Collections: After approximately 80% of control larvae have completed tail resorption, or supply of field-collected surface waters have been depleted larvae will be staged and tail height and length measured. Animals from each treatment will be euthanized by immersion in 3-aminobenzoic acid ethyl ester (MS-222, 1g/L, SOP **AF-3-03**). Approximately 15 animals from each treatment will be placed in 10% NBF while approximately 5 animals from each treatment will be frozen for subsequent determination of AP content.

Section 15.6. Endpoint Analysis: Tail length and height will be measured and recorded on Day 0 and on the last day of exposure for subsequent analysis of tail resorption.

Justification and Impact on Study:

Section 14, Section 15.3, Section 15.5, Section 15.6. The length of exposure was extended due to the slow development of all larvae, including control larvae. It has been previously established that larvae develop at different rates. Unfortunately, few of the larvae used in this trial with laboratory prepared solutions and reference and contaminated surface waters collected in July 2001 have exhibited forelimb emergence or complete tail resorption as of Day 19. Even with extending this trial it is unlikely that 80% of control larvae will complete tail resorption prior to the depletion of our July 2001 field-collected water supply.

Submitted by: Signature: Wanda L. Goleman Date: 2/1/02

Authorized by: Study Director: Wanda L. Goleman Date: 2/1/02

Received by: Quality Assurance Unit: [signature] Date: 2/5/02

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Check One: __X_ Protocol ____ SOP ____ Other _____

Title: The effects of contaminated and reference surface waters on metamorphosis in *Xenopus laevis* using a modified US EPA Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC, USEPA, 1998)- tier 1 tail resorption assay.
Dated: March 21, 2001
Document # (if appropriate): T9700.2; XEN-01-02; Change in Study No. 1; Change in Study No. 2; Change in Study No. 3; Change in Study No. 4
Page #(s): Protocol: 5, 6,7, 12
Change in Study No. 1: 2
Change in Study No. 2: 1
Change in Study No. 3: 2
Change in Study No. 4: 2
Section #: 12, 14, 15.2, 15.3, 15.5, 15.6

Text to reference: Section 12. TEST ANIMALS (Where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test system):
Species: South African Clawed Frog, *Xenopus laevis*
Strain: wild type
Age: Larval stages 55 - 66
Number: Approximately 580
Source: Laboratory colony and Xenopus Express, Homosassa, FL.

Section 14. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:
Approximately 20 *Xenopus larvae* (NF stages 55-66, Nieuwkoop and Faber, 1967) will be exposed to one of two laboratory prepared concentrations of AP in FETAX medium or FETAX medium alone until approximately 80% of control larvae have completed tail resorption, or supply of field-collected surface waters (concurrent study) have been depleted. Each treatment will be performed in triplicate. This will give approximately 20 larvae per treatment, for a study total of approximately 180 larvae. The AP concentration to be used represent high and low concentrations reported in April 1999 for effluent from the burning ground No. 3 ground water treatment plant (GWTP) located at the Longhorn Army Ammunition plant (LHAAP) in Karnack, TX.

Surface waters will be collected (SOP **AQ-3-02**) from 2 contaminated and 2 reference sites located at LHAAP approximately bimonthly, if possible, from February to July

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2001. Each collection site will be referenced by its full name or a 4-letter abbreviation. Identified contaminated collection sites are holding pond (HOLP) and Harrison Bayou catfish pond (HBCP). Identified reference collection sites are holding pond reference ditch (HPRD) and Star Ranch Pond (STAR). Waters will be collected by hand in clean 4-L glass bottles and will be kept cool during transport to our laboratory where they will be stored in a walk-in cooler at 4° C. Approximately 20 *Xenopus* larvae (NF stages 55-66, Nieuwkoop and Faber, 1967) will be exposed to each field-collected surface water or FETAX medium until approximately 80% of control larvae have completed tail resorption, or supply of field-collected surface waters have been depleted. Data collected during earlier assays using surface waters collected during April and August 2000 will also be included in the statistical analysis. This will give approximately 20 larvae per collection site X 4 collection sites X 6 months plus 20 control animals per collection, for a study total of approximately 520 larvae.

Section 15.3. TEST MATERIAL APPLICATION: Route/Method of Application: Larvae will be exposed to AP in the beaker medium. Larvae will be maintained in 1000-mL of the test solution in 2000-mL glass beakers maintained at 22° C ± 2° C until approximately 80% of control larvae have completed tail resorption, or supply of field-collected surface waters have been depleted. Method of application will be immersion. Route of exposure will be via dermal, oral, and respiratory exposure as the chemical will be in the beaker medium.

Section 15.5. ANIMAL SACRIFICE AND SAMPLE COLLECTIONS: After approximately 80% of control larvae have completed tail resorption, or supply of field-collected surface waters have been depleted larvae will be staged and tail height and length measured. Animals from each treatment will be euthanized by immersion in 3-aminobenzoic acid ethyl ester (MS-222, 1g/L, SOP **AF-3-03**). Approximately 15 animals from each treatment will be placed in 10% NBF while approximately 5 animals from each treatment will be frozen for subsequent determination of AP content.
Section 15.6. Endpoint Analysis: Tail length and height will be measured and recorded on Day 0 and on the last day of exposure for subsequent analysis of tail resorption.

Change in Document: Section 12. TEST ANIMALS (Where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test system):
Species: South African Clawed Frog, *Xenopus laevis*
Strain: wild type
Age: Larval stages 55 - 66
Number: Approximately 1008
Source: Laboratory colony and Xenopus Express, Homosassa, FL.

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Section 14. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL: Approximately 20 *Xenopus larvae* (NF stages 55-66, Nieuwkoop and Faber, 1967) were exposed to one of two laboratory prepared concentrations of AP in FETAX medium or FETAX medium alone for 36 or 45 days. Each treatment was performed in triplicate. Additionally the first trial failed within 3 days of initialization. This gave approximately 180 larvae per treatment plus 100 control animals, for a study total of 460 larvae. The AP concentration used represented high and low concentrations reported in April 1999 for effluent from the burning ground No. 3 ground water treatment plant (GWTP) located at the Longhorn Army Ammunition plant (LHAAP) in Karnack, TX.

Surface waters were collected (SOP AQ-3-02) from 2 contaminated and 2 reference sites located at LHAAP during April and August of 2000 and February, April, and July of 2001. A 14 d modified assay using 14 NF stage 60 larvae per beaker and 50% surface waters was run with surface waters collected in April 2000. Surface waters were diluted initially due to concern of a possible increase in mortality from the elevated salinity and conductivity. A 21 d study with 20 NF stage 55 larvae per beaker and 100% surface waters was run with the surface waters collected during August 2000. Contaminated sites identified at LHAAP were the INF treaty (holding) pond (HOLP) and Harrison Bayou catfish pond (HBCP). Identified reference collection sites were the holding pond reference ditch (HPRD) and Star Ranch Pond (STAR). Additionally, one assay was run using waters collected in August 2000 from another contaminated and closely matched reference site identified as building 25-C (B25C). However, since there was only one collection possible from B25C, these data were not included in statistical analyses. Surface waters were collected by hand in clean 4-L glass bottles, transported to Texas Tech University, and stored in a walk-in cooler at approximately 4 °C in our laboratory until use. Twenty *Xenopus* larvae, NF stage 55, were exposed to each field-collected water or FETAX medium, for 14, 21, 36, or 45 d. This gave either 14 or 20 larvae per collection site X 3 or 4 collection sites X 5 collections plus at least 20 control animals per collection, for a study total of approximately 548 larvae. (Note: February 2001 and July 2001 surface waters exposures were run concurrently with laboratory solution exposures. Therefore, the FETAX control animals were counted in the total for the laboratory solutions exposures.)

Section 15.3. TEST MATERIAL APPLICATION: Route/Method of Application: Larvae were exposed to perchlorate in the beaker medium. Larvae were maintained in 1 L of the test solutions in 2 L glass beakers maintained at 22 $\pm$ 2° C for 14, 21, 36, or 45 d. All solutions were changed every 3 d, with minor exceptions, as stated in the SOP for *Xenopus* husbandry (SOP AF-1-01). Method of application was immersion. Route of exposure was via dermal, oral, and respiratory exposure as the chemical was in the beaker medium.

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Section 15.5. ANIMAL SACRIFICE AND SAMPLE COLLECTIONS: After exposure larvae were staged with snout-vent length, hindlimb length, and tail height and length measured. Animals from all treatments were euthanized by immersion in MS-222 (1g/L, SOP AF-3-03). Approximately 15 animals from each treatment were placed in 10% NBF while 5 animals from each treatment were frozen for subsequent determination of perchlorate content.

Section 15.6. ENDPOINT ANALYSIS: Tail length and height were measured and recorded on Day 0 and on the last day of exposure for subsequent analysis of tail resorption. Snout-vent and hindlimb lengths were also recorded on Day 0 and on the last day of exposure.

Justification and Impact on Study:

Section 12. The total number of animals used in this study increased dramatically due to the inclusion of preliminary studies as well as one failed trial.

Section 14, Section 15.2, Section 15.3, Section 15.5, Section 15.6. The length of exposure varied due to the slow development of all larvae, including control and reference animals. It was previously established that larvae develop at different rates. We have found this assay lacking the sensitivity to detect the more subtle effects of perchlorate on thyroid hormone synthesis. Even with extending trials up to 45 d all control larvae did not reach NF stage 66. Although the final results of this study should not be affected, the modified experiment is not very useful as a short-term assay.

Submitted by: Signature: _____ Date: _____

Authorized by: Study Director: _____ Date: _____

Received by: Quality Assurance Unit: _____ Date: _____

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A FINAL REPORT

ENTITLED

LETHAL CONCENTRATION DETERMINATION OF SODIUM PERCHLORATE AND AMMONIUM CHLORIDE ON *XENOPUS LAEVIS* EGGS AND DEVELOPING JUVENILES DURING A 5 DAY EXPOSURE

STUDY NUMBER:	XEN-01-01
SPONSOR:	Strategic Environmental Research and Development Program (SERDP) 1155 Herndon Parkway, Suite 900 Herndon, Virginia 20170
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University Texas Tech University Health Sciences Center Box 41163 Lubbock, TX 79409-1163
TEST SITE:	The Institute of Environmental and Human Health Texas Tech University Texas Tech University Health Sciences Center Box 41163 Lubbock, TX 79409-1163
ANALYTICAL TEST SITE:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Center Box 41163 Lubbock, Texas 79409-1163
RESEARCH INITIATION:	February 15, 2001
RESEARCH COMPLETION:	November 26, 2001

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List of Tables and Figures

GOOD LABORATORY PRACTICES STATEMENT

Project XEN-01-01, entitled " Lethal concentration determination of sodium perchlorate and ammonium chloride on *Xenopus laevis* eggs and developing juveniles during a 5 day exposure", was performed whenever possible in the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Noted exceptions are as follows:

Submitted By:

James A. Carr, Ph.D

March 28, 2002
Date

QUALITY ASSURANCE STATEMENT

This study was conducted under the Institute of Environmental and Human Health's Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Any changes in protocol and SOPs were documented in writing and signed by the study director.

The Quality Assurance Officer verbally notified the Study Director of all findings at the time of the inspection. Written audit reports were also submitted to the Study Director and Test Facility Management. Audits were performed for the following phases of the project:

Auditable Research Phase / Activity	Audit Dates		Date written report submitted to Study Director	Date written report submitted to Management
	Start	End		
Final Report and Raw Data Review	2/20/02	3/12/02	3/19/02	3/19/02

Submitted By:

Ryan Bounds
Quality Assurance Manager

3/28/02
Date

1.0 DESCRIPTIVE STUDY TITLE:
Lethal concentration determination of sodium perchlorate and ammonium chloride on *Xenopus laevis eggs* and developing juveniles during a 5 day exposure.

2.0 STUDY NUMBER: XEN-01-01

3.0 SPONSOR:
Strategic Environmental Research
and Development Program (SERDP)
1155 Herndon Parkway, Suite 900
Herndon, Virginia 20170

4.0 TESTING FACILITY NAME & ADDRESS:
The Institute of Environmental and Human Health
Texas Tech University
Texas Tech University Health Sciences Center
Box 41163
Lubbock, TX 79409-1163

5.0 EXPERIMENTAL START & TERMINATION DATES:
Start Date: February 15, 2001
Termination Date: November 26, 2001

6.0 KEY PERSONNEL:
James A. Carr, Co-Principle Investigator
Wanda Goleman, Study Director
Todd Anderson, Analytical Chemist
Ryan Bounds, Quality Assurance Manager
Ken Dixon, Statistical/Modeling support
Ron Kendall, Principal Investigator / Testing Facility Management

7.0 STUDY OBJECTIVES / PURPOSE:

To determine the lethal concentrations of sodium perchlorate and ammonium chloride on *Xenopus laevis* when exposed for 5 days, beginning within 24 hours of when the eggs are laid.

8.0 STUDY SUMMARY

Embryonic *X. laevis* were exposed to a single concentration of sodium perchlorate, ammonium chloride, or plain FETAX medium for 5 d beginning < 24hr after fertilization. Ammonium chloride was significantly more lethal than sodium perchlorate, the respective 5 d LC50s were 118 ppm and > 1,220 ppm. The fact that the LC50 for ammonium perchlorate (Goleman et al., 2002) is closer to that of ammonium chloride than sodium perchlorate suggest that ammonium ions contribute to the lethality of ammonium perchlorate.

9.0 TEST MATERIALS:

Test Chemical name: Sodium Perchlorate (SP)
CAS number : 7601-89-0
Characterization: 98% (Assay)
Source: Aldrich Chemical Company

Test Chemical name: Ammonium Chloride (AC)
CAS number: 12125-02-9
Characterization: 99.99% (Assay)
Source: Aldrich Chemical Company

Reference Chemical name: de-ionized water
CAS number: not applicable
Characterization: FETAX (Frog Embryo Teratogenesis Assay- *Xenopus*) medium, a mixture of reagent grade salts, prepared in 100% ultrapure water. The quality of the water will be confirmed by analytical tests.
Source: City tap water that has been run through reverse osmosis and a de-ionizer to convert it to ultrapure water and contains reagent grade salts in the following concentrations (Sunderman et al., 1991): NaCl, 10.7 mM; $NaHCO_3$, 1.14 mM; KCl, 0.4 mM; $CaCl_2$, 0.14 mM; $CaSO_4$, 0.35 mM; $MgSO_4$, 0.62 mM.

10.0 JUSTIFICATION OF TEST SYSTEM

Ionic perchlorate alters calcium balance in fishes and amphibians (Luttgau et al., 1983; Thevenod et al., 1992; Jong et al., 1997) as well as other vertebrates. Calcium is a ubiquitous chemical messenger that is involved in the regulation of cellular function. Endocrine glands require calcium for the normal secretion of hormones and therefore contaminant-induced disruption of calcium balance can lead to systemic endocrine disruption. Perchlorate is also known to prevent intake of iodine from water or food and

thus it is goitrogenic (thyroid gland inhibitor) in many animals including fishes and amphibians (Miranda et al., 1996; Manzon and Youson, 1997). Because of the important role played by hormones in animal development and reproduction, endocrine disruption is likely to lead to serious impairments in growth, reproductive fitness, and consequently, amphibian and wildlife population stability as well as human health. Although lethality data already exists for ammonium perchlorate (AP), the relative contribution of ammonium to the lethality of AP has not been tested.

X. laevis are a widely used animal model in basic toxicological, developmental, and reproductive research. Also, there is a considerable database already available for this species. They represent a vertebrate class, amphibians, with distinct developmental and physiological adaptations to their aquatic environment. They are also easily and economically maintained and bred in the laboratory. Therefore, this species is ideally suited to examine the sublethal effects of contaminants such as AP on aquatic fauna.

11.0 TEST ANIMALS (Where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test system):
Species: South African Clawed Frog, *Xenopus laevis*
Strain: Wild type
Age: Eggs and larvae
Number: Approximately 7,854
Source: Laboratory colony and *Xenopus* Express (Homosassa, FL).

12.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:
Each container was labeled as indicated in SOP IN-3-06, which includes genus and species name; common name; project name, number, and start date; and the name of the person responsible for animal care. Each container was labeled to include sex of the individuals (if appropriate), date eggs were laid/hatched (if applicable), the name of the test substance and its concentration amount.

13.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:
X. laevis eggs (N=51) were exposed to one of ten concentrations of SP and ten concentrations of AC both in duplicate. Two additional beakers contained eggs (N=51) exposed to FETAX medium. Exposures were terminated after five days. This gave 102 eggs/larvae per treatment, for a study total of approximately 7,854 eggs/larvae.

14.0 METHODS:

14.1 Test System Acquisition, Quarantine, Acclimation

14.2 For each trial, three adult male and three adult female *X. laevis* were obtained from *Xenopus* Express (Homosassa, FL). Refer to SOP AQ-1-06 for details on routine *X. laevis* husbandry. They were maintained in 45-L glass tanks containing 18 L of ultrapure water for 1-2 days at 20° C on a 12L:12D light regimen. Male and female *X. laevis* were maintained separately for 7 d before breeding. Please refer to SOP AQ-1-04 for details on *X. laevis* breeding.

14.3 Test Condition Establishment

Naturally fertilized eggs were used. They were obtained from three pairs of adults who had been artificially induced to spawn (see *X. laevis* husbandry SOP AQ-1-04). These eggs were collected and examined under a microscope for viability (AQ-1-07). Viable eggs were counted into 42 groups of 17 from each female. A group of 17 randomly chosen eggs from each of 3 females was added to each beaker with test concentrations of SP, AC, or FETAX medium. Eggs and tadpoles up to five days of age were held in 250-mL glass beakers containing either test concentrations or control solution. Unfertilized eggs were disposed of appropriately. Any undeveloped eggs through the exposure period were noted. Each beaker/tank was labeled as indicated in SOP IN-3-06, which includes genus and species name, common name, project name, number, and start date, sex of the individuals (if appropriate), date eggs were laid/hatched (if applicable), and the name of the person responsible for animal care.

14.3.5 Adults were induced to spawn according to SOP AQ-1-04.

14.3.6 Eggs were sorted randomly into groups of 17 viable eggs from each female. Groups of 51 eggs, representing 17 randomly chosen eggs from each of 3 females, were placed into appropriately labeled beakers containing a single concentration of test solution in FETAX medium or FETAX medium alone.

14.4 Test Material Application

Test material was premixed to appropriate concentrations and added to the appropriately labeled glass aquaria (see section 15.2). Larvae were added to 21 L glass aquaria containing 8 L of test or reference solution. A 50% solution change containing the identical concentration of test substance was performed daily.

Rates/concentrations: 0, 1E-7, 1E-6, 1E-5, 1E-4, 3E-4, 5E-4, 1E-3, 3E-3, 5E-3, 1E-2 M.

Or:

Sodium perchlorate (mg/L): 1,220, 610, 366, 122, 61, 37, 12.2, 1.22, 0.122, 0.0122

Ammonium chloride (mg/L): 530, 265, 159, 53, 26.5, 15.9, 5.3, 0.53, 0.053, 0.0053

Frequency: Constant exposure for 5-d.

Route/Method of Application:

Eggs and larvae were exposed to SP or AC in the beaker medium. Eggs were maintained in 100 mL of the test solution in 250 mL beakers maintained in an incubator acclimated to 20° C. Test and reference solutions were changed every 72 hrs as stated in the SOP for *X. laevis* husbandry (AQ-1-06). Medium containing the identical concentration of test substance was added back to each

beaker as needed to maintain test conditions. Method of application was immersion. Route of exposure was via dermal, oral, and respiratory exposure as the chemical was in the beaker/aquaria medium.

Justification for Exposure Route:
X. laevis are fully aquatic as larvae and as adults.

Exposure Verification:
Samples of test and reference solutions were analyzed for SP or AC content. These measurements were taken after 72 hrs and upon termination of the exposure. Samples from each beaker containing SP were measured for perchlorate amounts according to the guidelines set forth in SOPs AC-2-11 and AC-1-01. Samples from each beaker containing AC were measured for ammonium amounts according to the guidelines set forth in SOP IN-4-12. At the end of the study remaining tadpoles were euthanized and frozen for possible contaminant analysis at a later date.

15.6 Test System Observation

Beginning on the day of hatch, hatching success (# unhatched eggs/total # eggs), % deformities (# showing bent tails, asymmetric tails/total hatched), edema (% showing distention of body with fluid/total hatched), and abnormal swimming (% showing abnormal swimming/total) were noted daily for each test and reference solution. For free-swimming larvae, % mortality (#dead larvae/#hatched), percent showing deformities, percent displaying abnormal swimming behavior were noted every day. Dead animals were removed and preserved in 10% formalin.

15.7 Animal Sacrifice and Sample Collections

Each trial was terminated after 5-d. Remaining tadpoles were immersed in MS-222 (3-aminobenzoic acid ethyl ester, 0.1% solution) according to AQ-1-03.

15.8 Endpoint Analysis

Hatching success, deformities (bent tails, asymmetric tails), edema (distention of body with fluid), and abnormal swimming were noted for hatchlings. Percent mortality (#dead/#hatched), deformities, and abnormal swimming behavior was recorded for larvae.

16. STATISTICAL METHODS:

LC_{50}s were calculated independently for each trial by the moving angle average method or the probit method using SoftTox ∞ software (ChemSW, Fairfield, CA, USA)..

17. PROTOCOL CHANGES / REVISIONS:

See attached change in study documentation forms.

18. RESULTS:

A total of four trials were conducted between February and November 2001. Because of high mortality in the controls, only data from the third and fourth trials is presented here. As shown in Tables 1 and 2, ammonium chloride was much more lethal than sodium perchlorate at the concentrations tested. The LC50s for ammonium chloride determined for trials 3 and 4 were 80 ppm and 155 ppm respectively, an average of 118 ppm. In contrast, the LC50s for sodium perchlorate from the two trials were greater than 1,220 ppm, as concentrations of sodium perchlorate up to 1,220 ppm failed to kill at least 50% of the embryos (Tables 1 and 2). Ammonium chloride also caused a greater incidence of bent tails and abnormal swimming compared to sodium perchlorate (Table 3). The incidence of abnormal swimming was 40-90% in tadpoles exposed to 139-530 ppm ammonium chloride compared to roughly 10 % of tadpoles exposed to the same concentrations of sodium perchlorate. Likewise, the incidence of bent tails was 35-73% in tadpoles exposed to 139-530 ppm ammonium chloride compared to roughly 7-10 % of tadpoles exposed to the same concentrations of sodium perchlorate. Interestingly, the incidence of edema was lower in the controls and ammonium chloride treated groups compared to the sodium perchlorate exposed animals (Table 3).

Table 1. Percent Hatching and Mortality in Larval *X. laevis* Exposed to Sodium Perchlorate or Ammonium Chloride for 5-d during Trial 3.

Sodium Perchlorate						
Nominal (M)	Nominal (ppm)	Perchlorate[a] (ppm)	NH3-N[b] (ppm)	N	Hatch[c]	Mortality[d]
1E –2	1,220	1,418	1.22	34	88.2 (30)	29.4 (10)
5E –3	610	ND	1.13	34	97.1 (33)	14.7 (5)
3E –3	366	297	1.37	34	97.1 (33)	11.8 (4)
1E –3	122	ND	1.34	34	94.1 (32)	14.7 (5)
5E –4	61.0	ND	1.57	34	97.1 (33)	8.82 (3)
3E –4	37.0	ND	1.46	34	94.1 (32)	14.7 (5)
1E –4	12.0	ND	1.27	34	100 (34)	2.94 (1)
1E –5	1.22	1.09	1.72	34	97.1 (33)	5.88 (2)
1E –6	0.12	ND	1.57	34	97.1 (33)	11.8 (4)
1E –7	0.01	ND	1.37	34	100 (34)	2.94 (1)

Ammonium Chloride						
Nominal (M)	Nominal (ppm)	Perchlorate (ppm)	NH3-N (ppm)	N	Hatch	Mortality
1E –2	530	ND	76.0	N	79.4 (27)	70.59 (24)
5E –3	265	ND	42.3	34	94.1 (32)	55.88 (19)
3E –3	159	ND	23.5	34	97.1 (33)	14.71 (5)
1E –3	53.0	ND	7.49	34	100 (34)	11.76 (4)
5E –4	27.0	ND	2.91	34	97.1 (33)	2.94 (1)
3E –4	16.0	ND	2.67	34	97.1 (33)	8.82 (3)
1E –4	5.30	ND	1.85	34	100 (34)	5.88 (2)
1E –5	0.53	ND	1.72	34	97.1 (33)	5.88 (2)
1E –6	0.05	ND	1.71	34	100 (34)	0.00 (0)
1E –7	0.01	ND	1.53	34	97.1 (33)	2.94 (1)
FETAX[e]			1.30	34		

[a]Average measured from beaker water samples 3/28/01. [b]Measured as ammonia nitrogen on 4/02/01. [c]Calculated as a percent of total eggs. Number hatching is presented in parentheses. [d]Calculated as a percent of total eggs. Number dead is presented in parentheses. [e]FETAX (Frog Embryo Teratogenic Assay – *Xenopus*; Dawson and Bantle, 1987).

Table 2. Percent Hatching and Mortality in Larval *X. laevis* Exposed to Sodium Perchlorate or Ammonium Chloride for 5-d during Trial 4.

Sodium Perchlorate						
Nominal (M)	Nominal (ppm)	Perchlorate[a] (ppm)	NH3-N[b] (ppm)	N	Hatch[c]	Mortality[d]
1E –2	1,220	1,071	1.26	102	95.1 (97)	10.8 (11)
5E –3	610	554	1.06	102	89.2 (91)	11.8 (12)
3E –3	366	332	1.14	102	88.2 (90)	11.8 (12)
1E –3	122	105	1.18	102	84.3 (86)	17.7 (18)
5E –4	61.0	52.1	1.48	102	90.2 (92)	14.7 (15)
3E –4	37.0	35.2	1.36	102	90.2 (92)	12.8 (13)
1E –4	12.0	12.3	1.36	102	85.3 (87)	16.7 (17)
1E –5	1.22	2.53	1.26	102	86.3 (88)	14.7 (15)
1E –6	0.12	0.18	1.50	102	84.3 (86)	18.6 (19)
1E –7	0.01	0.02	1.57	102	89.2 (91)	18.6 (19)

Ammonium Chloride						
Nominal (M)	Nominal (ppm)	Perchlorate (ppm)	NH3-N (ppm)	N	Hatch	Mortality
1E –2	530	0.00	59.2	102	81.4 (83)	100 (102)
5E –3	265	0.00	51.4	102	60.8 (62)	100 (102)
3E –3	159	0.00	48.1	102	90.2 (92)	39.2 (40)
1E –3	53.0	0.00	18.7	102	89.2 (91)	15.7 (16)
5E –4	27.0	0.00	9.03	102	88.2 (90)	15.7 (16)
3E –4	16.0	0.00	8.42	102	87.3 (89)	15.7 (16)
1E –4	5.30	0.00	2.83	102	80.4 (82)	20.6 (21)
1E –5	0.53	0.00	1.42	102	82.4 (84)	19.6 (20)
1E –6	0.05	0.00	1.28	102	88.2 (90)	16.7 (17)
1E –7	0.01	0.00	1.32	102	89.2 (91)	11.8 (12)
FETAX[e]		0.00	1.36	102	90.2 (92)	12.8 (13)

[a]Average measured from beaker water samples collected 11/24/01-11/26/01. [b]Measured from beaker water samples as ammonia nitrogen on 11/26/01. [c]Calculated as a percent of total eggs. Number hatching is presented in parentheses. [d]Calculated as a percent of total eggs. Number dead is presented in parentheses. [e]FETAX (Frog Embryo Teratogenic Assay – *Xenopus*; Dawson and Bantle, 1987).

Table 3. Developmental Abnormalities in Larval *X. laevis* Exposed to Sodium Perchlorate or Ammonium Chloride for 5-d during trial 4.

Sodium Perchlorate				
Nominal (M)	Nominal (ppm)	Bent tails (%)	Edema (%)	Abnormal Swimming (%)
1E –2	1,220	19.59 (19)	15.46 (15)	15.46 (15)
5E –3	610	7.69 (7)	13.19 (12)	10.99 (10)
3E –3	366	11.11 (10)	13.33 (12)	10.00 (9)
1E –3	122	6.98 (6)	12.79 (11)	10.47 (9)
5E –4	61.0	2.17 (2)	9.78 (9)	2.17 (2)
3E –4	37.0	5.43 (5)	6.53 (6)	9.78 (9)
1E –4	12.0	6.90 (6)	2.30 (2)	5.75 (5)
1E –5	1.22	7.95 (7)	5.68 (5)	10.23 (9)
1E –6	0.12	3.49 (3)	6.98 (6)	6.98 (6)
1E –7	0.01	3.30 (3)	3.30 (3)	5.49 (5)
Ammonium Chloride				
Nominal (M)	Nominal (ppm)	Bent tails (%)	Edema (%)	Abnormal Swimming (%)
1E –2	530	43.37 (36)	0.00 (0)	43.37 (36)
5E –3	265	35.48 (22)	0.00 (0)	46.77 (29)
3E –3	159	72.83 (67)	0.00 (0)	90.22 (83)
1E –3	53.0	15.38 (14)	1.10 (1)	25.27 (23)
5E –4	27.0	6.67 (6)	0.00 (0)	5.56 (5)
3E –4	16.0	3.37 (3)	1.12 (1)	3.37 (3)
1E –4	5.30	9.76 (8)	0.00 (0)	6.10 (5)
1E –5	0.53	4.76 (4)	0.00 (0)	2.38 (2)
1E –6	0.05	7.78 (7)	0.00 (0)	5.56 (5)
1E –7	0.01	3.30 (3)	0.00 (0)	3.30 (3)
FETAX[a]		5.43 (5)	0.00 (0)	3.26 (3)

[a]FETAX (Frog Embryo Teratogenic Assay – *Xenopus*; Sunderman et al., 1991)

19. DISCUSSION

Work in our laboratory (Goleman et al., 2002) on ammonium perchlorate (AP) found five- and seventy-day LC_{50} values of 510 ± 36 mg/L and 223 ± 13 mg /L), respectively. In that previous study we did not directly assess the contribution of ammonium ions to the lethality of AP, but data contained in this report indicates a mean 5-d LC_{50} of 118 mg/L for ammonium chloride and an LC_{50} of greater that 1,200 ppm for sodium perchlorate using the same protocol described Goleman et al. (2002). The LC_{50} for ammonium chloride determine in the present study (118 ppm) is comparable to the 4-d LC_{50} of 127.5 mg/L reported for ammonium chloride in *X. laevis* (Schuytema and Nebeker, 1999). The fact that the 5-d LC_{50} calculated for AP is closer to that of ammonium chloride than to that of sodium perchlorate suggests that ammonium ions may significantly contribute to the lethality of AP. Our findings also suggest that ammonium ions contribute to the effects of AP on abnormal swimming. Goleman et al. (2002) found that AP at a concentration of 425 ppm resulted in 55-88% abnormal swimming. Similar concentrations of sodium perchlorate do not significantly effect the incidence of abnormal swimming (Table 3).

20. STUDY RECORDS AND ARCHIVE:

Study Records will be maintained at The Institute of Environmental and Human Health (TIEHH) Archive for a minimum of one year after study completion date.

21. REFERENCES:

Dawson DA, Bantle JA. (1987). Development of a reconstituted water medium and preliminary validation of the frog embryo teratogenesis assay-*Xenopus* (FETAX). J. Appl. Toxicol. 7:237-244.

Goleman,W.L., Urquidi, L.J., Anderson, T.A., Kendall, R.J., Smith E.E., and Carr, J.A. (2002). Environmentally relevant concentrations of ammonium perchlorate inhibit development and metamorphosis in *Xenopus laevis*. Environ. Toxicol. Chem. 21: 424-430.

Manzon, R.G. and Youson, J.H. (1997). The effects of exogenous thyroxine (T_4) on triiodothyronine (T_3), in the presence or absence of potassium perchlorate, on the incidence of metamorphosis and on serum T_4 and T_3 concentrations in larval sea lampreys (*Petromyzon marinus* L). Gen. Comp. Endocrinol. 106: 211-220.

Miranda, L.A., Pisano, A. and Casco, V. (1996). Ultrastructural study of thyroid glands of *Bufo arenarum* larvae kept in potassium perchlorate solution. Biocell 20: 147-153.

Schuytema, G.S. and Nebeker, A.V. (1999). Comparative effects of ammonium and nitrate compounds on Pacific treefrog and African clawed frog embryos. Arch. Environ. Contam. Toxicol. 36:200-206.

Siglin, J.C., Mattie, D.R., Dodd, D.E., Hildebrandt, P.K., Baker, W.H. (2000). A 90-day drinking water toxicity study in rats of the environmental contaminant ammonium perchlorate. Toxicol. Sci. 57:61-74.

Taylor, A.C. and Kollros, J..J. (1946). Stages in the normal development of *Rana pipens* larvae. Anat. Rec. 94: 7-23.

Urbansky, E.T. (1998). Perchlorate chemistry: implications for analysis and remediation. Bioremediation J. 2: 81-95.

22. APPENDICES:

Study Protocol
Changes to Study Documentation
- Change in Study No. 1
- Change in Study No. 2
- Change in Study No. 3
- Change in Study No. 4
- Change in Study No. 5

Study Phase Inspection Reports
- Changing of Test Material and Reference Solution
- Mixing and Application of Test Material

A STUDY PROTOCOL

ENTITLED

LETHAL CONCENTRATION DETERMINATION OF SODIUM PERCHLORATE AND AMMONIUM CHLORIDE ON *XENOPUS LAEVIS* EGGS AND DEVELOPING JUVENILES DURING A 5 DAY EXPOSURE

STUDY NUMBER: XEN-01-01

SPONSOR:

United States Air Force
IERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

TESTING FACILITY

Name/Address: The Institute of Environmental and Human Health
Texas Tech University
Texas Tech University Health Sciences Center
Box 41163
Lubbock, TX 79409-1163

Test Facility Management: Dr. Ronald J. Kendall

Study Director: Wanda Goleman

PROPOSED EXPERIMENTAL
START DATE FEBRUARY 15, 2001

1. **DESCRIPTIVE STUDY TITLE**: Lethal concentration determination of sodium perchlorate and ammonium chloride on *Xenopus laevis eggs* and developing juveniles during a 5 day exposure.

2. **STUDY NUMBER:** XEN-01-01

3. **SPONSOR:** United States Air Force United States Air Force
IERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

4. **TESTING FACILITY NAME & ADDRESS:**
The Institute of Environmental and Human Health
Texas Tech University
Texas Tech University Health Sciences Center
Box 41163
Lubbock, TX 79409-1163

5. **PROPOSED EXPERIMENTAL START & TERMINATION DATES:**
Start Date: February 15, 2001
Termination Date: August 31, 2001

6. **KEY PERSONNEL:**
James A. Carr, Co-Principle Investigator
Wanda Goleman, Study Director
John Blevins, Graduate Research Assistant
Todd Anderson, Analytical Chemist
Ryan Bounds, Quality Assurance Manager
Ken Dixon, Statistical/Modeling support
Ron Kendall, Principal Investigator / Testing Facility Management

7. DATED SIGNATURES

Wanda L. Goleman	2/15/01	Wanda Goleman Study Director
James Carr	2/15/01	Dr. James Carr Co-Principle Investigator
Todd Anderson	2-19-01	Dr. Todd Anderson Analytical Chemist
	2/16/01	Ryan Bounds Quality Assurance Manager
	2/19/01	Dr. Ken Dixon Statistician
Ron Kendall	2/19/01	Dr. Ron Kendall Principle Investigator/ Testing Facility Management

8. REGULATORY COMPLIANCE STATEMENT

Quality Control and Quality Assurance

This study will be conducted in accordance with established Quality Assurance program guidelines and in compliance, where appropriate and possible, with Good Laboratory Practice Standards (40 CFR Part 160, August 17, 1989).

Document Control Statement

This document is considered proprietary to and the project sponsor. Do not copy, quote or distribute. For access to this document or authority to release or distribute, please write to:

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Lubbock, Texas 79409

9. STUDY OBJECTIVES / PURPOSE:

To determine the lethal concentrations of sodium perchlorate and ammonium chloride on *Xenopus laevis* when exposed for 5 days, beginning within 24 hours of when the eggs are laid.

10. TEST MATERIALS:

Test Chemical name: Sodium Perchlorate (SP)
CAS number : 7601-89-0
Characterization: 98% (Assay)
Source: Aldrich Chemical Company

Test Chemical name: Ammonium Chloride (AC)
CAS number: 12125-02-9
Characterization: 99.99% (Assay)
Source: Aldrich Chemical Company

Reference Chemical name: de-ionized water
CAS number: not applicable
Characterization: FETAX (Frog Embryo Teratogenesis Assay- *Xenopus*) medium, a mixture of reagent grade salts, prepared in 100% ultrapure water. The quality of the water will be confirmed by analytical tests.
Source: City tap water that has been run through reverse osmosis and a de-ionizer to convert it to ultrapure water and contains reagent grade salts in the following concentrations (Sunderman et al., 1991): NaCl, 10.7 mM; $NaHCO_3$, 1.14 mM, KCl, 0.4 mM; $CaCl_2$, 0.14 mM; $CaSO_4$, 0.35 mM, $MgSO_4$, 0.62 mM.

11. JUSTIFICATION OF TEST SYSTEM

Ionic perchlorate alters calcium balance in fishes and amphibians (Luttgau et al., 1983; Thevenod et al., 1992; Jong et al., 1997) as well as other vertebrates. Calcium is a ubiquitous chemical messenger that is involved in the regulation of cellular function. Endocrine glands require calcium for the normal secretion of hormones and therefore contaminant-induced disruption of calcium balance can lead to systemic endocrine disruption. Perchlorate is also known to prevent intake of iodine from water or food and thus it is goitrogenic (thyroid gland inhibitor) in many animals including fishes and amphibians (Miranda et al., 1996; Manzon and Youson, 1997). Because of the important role played by hormones in animal development and reproduction, endocrine disruption is likely to lead to serious impairments in growth, reproductive fitness, and consequently, amphibian and wildlife population stability as well as human health. Although lethality data already exists for ammonium perchlorate (AP), the relative contribution of ammonium to the lethality of AP has not been tested.

X. laevis are a widely used animal model in basic toxicological, developmental, and reproductive research. Also, there is a considerable database already available for this species. They represent a vertebrate class, amphibians, with distinct developmental and physiological adaptations to their aquatic environment. They are also easily and economically maintained and bred in the laboratory. Therefore, this species is ideally suited to examine the sublethal effects of contaminants such as AP on aquatic fauna.

12. TEST ANIMALS:

Species: South African Clawed Frog, *Xenopus laevis*
Strain: Wild type
Age: Eggs and larvae
Number: Approximately 2,142
Source: Laboratory colony and *Xenopus* Express (Homosassa, FL).

13. PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Each container will be labeled as indicated in TIEHH SOP **IN-3-06**, which includes genus and species name; common name; project name, number, and start date; and the name of the person responsible for animal care. Each container will also be labeled to include sex of the individuals (if appropriate), date eggs were laid/hatched (if applicable), the name of the test substance and its concentration amount.

14. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

X. laevis eggs (N=51) will be exposed to one of ten concentrations of SP and ten concentrations of AC both in duplicate. Two additional beakers will contain eggs (N=51) exposed to FETAX medium. Exposures will be terminated after five days. This will give 102 eggs/larvae per treatment, for a study total of 2,142 eggs/larvae.

15. METHODS:

15.1 Test System acquisition, quarantine, and acclimation

Three adult male and three adult female *X. laevis* will be obtained from *Xenopus* Express (Homosassa, FL). Refer to TIEHH SOP **AQ-1-06** for details on routine *X. laevis* husbandry. They will be maintained in 45-L glass tanks containing 18 L of ultrapure water for 1-2 days at 20° C on a 12L:12D light regimen. Male and female *X. laevis* will be maintained separately for 7 d before breeding. Please refer to SOP **AQ-1-04** for details on *X. laevis* breeding.

15.2 Test condition establishment

Naturally fertilized eggs will be used. They will be obtained from three pairs of adults who have been artificially induced to spawn (see *X. laevis* husbandry SOP **AQ-1-04**). These eggs will be collected and examined under a microscope for viability (**AQ-1-07**). Viable eggs will be counted into 42 groups of 17 from each female. A group of 17 randomly chosen eggs from each of 3 females will be added to each beaker with test concentrations of SP, AC, or FETAX medium. Eggs and tadpoles up to five days of age will be held in 250-mL glass beakers containing either test concentrations or control solution. Unfertilized eggs will be disposed of appropriately. Any undeveloped eggs through the exposure period will be noted. Each beaker/tank will be labeled as indicated in TIEHH SOP **IN-3-06**, which includes genus and species name, common name, project name, number, and start date, sex of the individuals (if appropriate), date eggs were laid/hatched (if applicable), and the name of the person responsible for animal care.

15.2a Adults will be induced to spawn according to SOP **AQ-1-04**.

15.2b Eggs will be sorted randomly into groups of 17 viable eggs from each female. Groups of 51 eggs, representing 17 randomly chosen eggs from each of 3 females, will be placed into appropriately labeled beakers containing a single concentration of test solution in FETAX medium or FETAX medium alone.

15.3 Test Material Application

Rates/concentrations: 0, 1E-7, 1E-6, 1E-5, 1E-4, 3E-4, 5E-4, 1E-3, 3E-3, 5E-3, 1E-2 M.

Frequency: Constant exposure for 5-d.

Route/Method of Application: Eggs and larvae will be exposed to SP or AC in the beaker medium. Eggs will be maintained in 100 mL of the test solution in 250 mL beakers maintained in an incubator acclimated to 20° C. Test and reference solutions will be changed every 72 hrs as stated in the SOP for *X. laevis* husbandry (**AQ-1-06**). Medium containing the identical concentration of test substance will be added back to each beaker as needed to maintain test conditions. Method of application will be immersion. Route of exposure will be via dermal, oral, and respiratory exposure as the chemical will be in the beaker/aquaria medium.

Justification for Exposure Route: *X. laevis* are fully aquatic as larvae and as adults.

Exposure Verification: Samples of test and reference solutions will be analyzed for SP or AC content. These measurements will be taken after 72 hrs and upon termination of the

exposure. Samples from each beaker containing SP will be measured for perchlorate amounts according to the guidelines set forth in SOPs **AC-2-11** and **AC-1-01.** Samples from each beaker containing AC will be measured for ammonium amounts according to the guidelines set forth in SOP **IN-4-12.** At the end of the study remaining tadpoles will be euthanized and frozen for possible contaminant analysis at a later date.

15.4 Test System Observation

Beginning on the day of hatch, hatching success (# unhatched eggs/total # eggs), % deformities (# showing bent tails, asymmetric tails/total hatched), edema (% showing distention of body with fluid/total hatched), and abnormal swimming (% showing abnormal swimming/total) will be noted daily for each test and reference solution. For free-swimming larvae, % mortality (#dead larvae/#hatched), percent showing deformities, percent displaying abnormal swimming behavior will be noted every day. Dead animals will be removed and preserved in 10% formalin.

15.5 Animal Sacrifice and Sample Collections

The experiment will be terminated after 5-d. Remaining tadpoles will be immersed in MS-222 (3-aminobenzoic acid ethyl ester, 0.1% solution) according to **AQ-1-03.**

15.6 Endpoint Analysis

Hatching success, deformities (bent tails, asymmetric tails), edema (distention of body with fluid), and abnormal swimming will be noted for hatchlings. Percent mortality (#dead/#hatched), deformities, and abnormal swimming behavior will be recorded for larvae.

16. PROPOSED STATISTICAL METHODS

Probit analysis will be used to determine the actual lethal concentration percentages.

17. REPORT CONTENT/RECORDS TO BE MAINTAINED:

Records to be maintained include:

- Incubator and water temperature, salinity, pH, and alkalinity will be collected.
- Date, time, and amount of feedings per tank/beaker will be recorded. Number of expired larvae removed prior to termination of exposure will be recorded, including each time, date, and beaker.
- Deformities and abnormal swimming behavior will be recorded daily prior to termination of the experiment.

Report content will also include presentation of data, interpretation, and discussion of the following end-points:

- LC_{50} and concentration-response curve
- Discussion of the relevance of the findings
- List of all SOPs used
- List of all personnel

18. RECORDS TO BE MAINTAINED / LOCATION:

The final report will be delivered to the Sponsor on or before March 11, 2002. Copies of all data, documentation, records, protocol information, as well as the specimens shall be sent to the Sponsor, or designated delivery point upon request. All data, the protocol and a copy of the final report shall be archived by the testing facility.

19. QUALITY ASSURANCE:

The Quality Assurance Unit will inspect the study at intervals to insure the integrity of the study. Written records will be maintained indicating but not limited to the following: date of inspection, study inspected, phase inspected, person conducting the inspection, findings and problems, recommended and taken action, and any scheduled reinspection. Any problems likely to effect study integrity shall be brought to the immediate attention of the Study Director. The Quality Assurance Unit will periodically submit written status reports on the study to management and the Study Director.

20. PROTOCOL CHANGES / REVISIONS:

All changes and/or revisions to the protocol, and the reasons therefore, shall be documented, signed and dated by the Study Director and maintained with the protocol and the Quality Assurance Unit.

21. REFERENCES:

Luttgau, H.C., Gottschalk, G., Kovacs, L., Fuxreiter, M. (1983). How perchlorate improves excitation-contraction coupling in skeletal muscle fibers Biophys. J. 43:247-249

Jong, D.S., Stroffekova, K., Heiny, J.A. (1997). A surface potential change in the membranes of frog skeletal muscle is associated with excitation-contraction coupling. J. Physiol.499:787-808.

Manzon, R.G. and Youson, J.H. (1997). The effects of exogenous thyroxine (T_4) on triiodothyronine (T_3), in the presence or absence of potassium perchlorate, on the incidence of metamorphosis and on serum T_4 and T_3 concentrations in larval sea lampreys (*Petromyzon marinus* L). *Gen. Comp. Endocrinol.* 106: 211-220.

Miranda, L.A., Pisano, A. and Casco, V. (1996). Ultrastructural study of thyroid glands of *Bufo arenarum* larvae kept in potassium perchlorate solution. *Biocell* 20: 147-153.

Nieuwkoop, P.D. and Faber, J. (1967). Normal table of *Xenopus laevis (Daudin)*, North Holland, Amsterdam.

Sunderman, F.W., Plowman, M.C. and Hopfer, S.M. (1991). Embryotoxicity and teratogenicity of cadmium chloride in *Xenopus laevis*, assayed by the FETAX procedure. Ann. Clin. Lab. Sci. 21: 381-391.

Thevenod, F. and Jones, S.W. (1992). Cadmium block of calcium current in frog sympathetic neurons. Biophys. J. 63:162-168.

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Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: x Amendment ____Deviation ____ Addendums

Document Reference Information
Check One: x Protocol ____ SOP ____ Other ____
Title: Lethal Concentration Determination of Sodium Perchlorate and Ammonium Chloride on *Xenopus laevis* eggs and Developing Juveniles During a 5 Day Exposure
Dated: March 9, 2001
Document # (if appropriate): T9700.2; XEN-01-01
Page #(s): 5
Section #: 12 and 14
Text to reference: Number: Approximately 2,142
This will give 102 eggs/larvae per treatment, for a total of 2,142 eggs, larvae

---

Change in Document: The total number of eggs/larvae to be used in the study will actually be 4,284

Justification and Impact on Study: During the first night of exposure to the separate chemicals, a problem occurred in the air lines, i.e. there were several beakers which did not receive enough oxygen. As a result, there was high mortality rates in several beakers. In order to determine whether this is a result of low dissolved oxygen or the effect of the chemical, the study should be conducted in duplicate.

Submitted by: Signature: _____ Date: 3/9/01

Authorized by: Study Director: _____ Date: 3/9/01

Received by: Quality Assurance Unit: _____ Date: 3/12/01

* Sequentially numbered in order of the date that the change is effective

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Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: X Amendment ____ Deviation ____ Addendums

Document Reference Information

Check One: X Protocol ____ SOP ____ Other ____

Title: Lethal concentration determination of sodium perchlorate and ammonium chloride on *Xenopus laevis eggs* and developing juveniles during a 5 day exposure
Dated: February 15, 2001
Document # (if appropriate): T9700.2; XEN-01-01; Change in Study #1
Page #(s): 5
Section #: 12, 14
Text to reference: Section 12. TEST ANIMALS: Number: Approximately 4,284
Section 14. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL: *X. laevis* eggs (N=51) will be exposed to one of ten concentrations of SP and ten concentrations of AC both in duplicate. Two additional beakers will contain eggs (N=51) exposed to FETAX medium. Exposures will be terminated after five days. This will give 102 eggs/larvae per treatment for each of two trials, for a study total of 4,284 eggs/larvae.

Change in Document: Section 12. TEST ANIMALS: Number: Approximately 6,426
Section 14. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL: *X. laevis* eggs (N=51) will be exposed to one of ten concentrations of SP and ten concentrations of AC both in duplicate. Two additional beakers will contain eggs (N=51) exposed to FETAX medium. Exposures will be terminated after five days. This will give 102 eggs/larvae per treatment for each of three trials, for a study total of 6,426 eggs/larvae.

Justification and Impact on Study: Section 12. The anticipated outcome of the heaviest concentrations of both ammonium chloride and sodium perchlorate were not achieved. There was a problem in mixing the test solutions causing almost no effect of exposure. A second repetition of the experiment is therefore necessary to validate the results.
Section 14. The anticipated outcome of the heaviest concentrations of both ammonium chloride and sodium perchlorate were not achieved. There was a problem in mixing the test solutions causing almost no effect of exposure. A second repetition of the experiment is therefore necessary to validate the results.

Submitted by: Signature: Wanda L Goleman Date: 6/12/01

Authorized by: Study Director: Wanda L. Goleman Date: 6/12/01

Received by: Quality Assurance Unit: Brian Bidwell Date: 3/19/02

* Sequentially numbered in order of the date that the change is effective

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Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: ____ Amendment __X__ Deviation ____ Addendums

Document Reference Information

Check One: __X__ Protocol ____ SOP ____ Other ____

Title: Lethal concentration determination of sodium perchlorate and ammonium chloride on *Xenopus laevis eggs* and developing juveniles during a 5 day exposure
Dated: February 15, 2001
Document # (if appropriate): T9700.2; XEN-01-01; Change in Study #2
Page #(s): 1
Section #: 12, 14
Text to reference: Section 12. TEST ANIMALS: Number: Approximately 6,426
Section 14. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL: *X. laevis* eggs (N=51) will be exposed to one of ten concentrations of SP and ten concentrations of AC both in duplicate. Two additional beakers will contain eggs (N=51) exposed to FETAX medium. Exposures will be terminated after five days. This will give 102 eggs/larvae per treatment for each of three trials, for a study total of 6,426 eggs/larvae.

Change in Document: Section 12. TEST ANIMALS: Number: Approximately 5,712
Section 14. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL: *X. laevis* eggs (N=51) will be exposed to one of ten concentrations of SP and ten concentrations of AC both in duplicate. Two additional beakers will contain eggs (N=51) exposed to FETAX medium. Exposures will be terminated after five days. This will give 102 eggs/larvae per treatment for each of three trials, for a study total of 5,712 eggs/larvae.

Justification and Impact on Study: Section 12. Only two of the three breeding pairs produced an adequate number of viable eggs. However, to avoid statistical confounding factors raised by a simple genetic difference between offspring of two pairs of adults, eggs from only one pair may be used. Only the previously planned number of eggs from a single female was placed into the experiment (n = 17 per beaker), reducing the total number of eggs/larvae used in trial 3.
Section 14. Only two of the three breeding pairs produced an adequate number of viable eggs. However, to avoid statistical confounding factors raised by a simple genetic difference between offspring of two pairs of adults, eggs from only one pair may be used. Only the previously planned number of eggs from a single female was placed into the experiment (n = 17 per beaker), reducing the total number of eggs/larvae used in trial 3.

Submitted by: Signature: Wanda L. Goleman Date: 6/12/01
Authorized by: Study Director: Wanda L. Goleman Date: 6/12/01
Received by: Quality Assurance Unit: Brian Birdwell Date: 3/19/02

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Form No. 014 Rev. 3.06/00
Project No.: T9700.2
***Change No.:** 4
Page: 1 **of** 1

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: ____ Amendment __X__ Deviation ____ Addendums

Document Reference Information

Check One: __X__ Protocol ____ SOP ____ Other _____

Title: Lethal concentration determination of sodium perchlorate and ammonium chloride on *Xenopus laevis eggs* and developing juveniles during a 5 day exposure
Dated: February 15, 2001
Document # (if appropriate): T9700.2; XEN-01-01
Page #(s): 6
Section #: 15.3
Text to reference: Section 15.3. Exposure Verification: Samples of test and reference solutions will be analyzed for SP or AC content. These measurements will be taken after 72 hrs and upon termination of the exposure. Samples from each beaker containing SP will be measured for perchlorate amounts according to the guidelines set forth in SOPs **AC-2-11** and **AC-1-01.** Samples from each beaker containing AC will be measured for ammonium amounts according to the guidelines set forth in SOP **IN-4-12.** At the end of the study remaining tadpoles will be euthanized and frozen for possible contaminant analysis at a later date.

Change in Document: Section 15.3. Exposure Verification: Samples of test and reference solutions will be analyzed for SP or AC content. These measurements will be taken after 72 hrs and upon termination of the exposure. Samples from each beaker containing SP will be measured for perchlorate amounts according to the guidelines set forth in SOPs **AC-2-11** and **AC-1-01.** Samples from each beaker containing AC will be measured for ammonium amounts according to the guidelines set forth in SOP **IN-4-12.** At the end of the study remaining tadpoles will be euthanized and stored in 10% neutral buffered formalin.

Justification and Impact on Study: Section 15.3. Remaining tadpoles were not frozen for perchlorate analysis. While analysis for exposure verification may still possible, it would be much more difficult and less accurate.

Submitted by: Signature: Wanda L. Goleman Date: 6/12/01

Authorized by: Study Director: Wanda L. Goleman Date: 6/12/01

Received by: Quality Assurance Unit: Brian Bilwell Date: 3/19/02

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Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: X Amendment ____Deviation ____ Addendums

Document Reference Information

Check One: X Protocol ____ SOP ____ Other _____

Title: Lethal concentration determination of sodium perchlorate and ammonium chloride on *Xenopus laevis eggs* and developing juveniles during a 5 day exposure

Dated: February 15, 2001

Document # (if appropriate): T9700.2; XEN-01-01; Change in Study 1; Change in Study 3; Change in Study 4

Page #(s): Protocol: 5, 6; Change in Study 1: 1; Change in Study 3: 1; Change in Study 4: 1

Section #: 12, 14, 15.3

Text to reference: Section 12. TEST ANIMALS: Number: Approximately 5,712
Section 14. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL: *X. laevis* eggs (N=51) will be exposed to one of ten concentrations of SP and ten concentrations of AC both in duplicate. Two additional beakers will contain eggs (N=51) exposed to FETAX medium. Exposures will be terminated after five days. This will give 102 eggs/larvae per treatment for each of three trials, for a study total of 5,712 eggs/larvae.
Section 15.3. Route/Method of Application: Eggs and larvae will be exposed to SP or AC in the beaker medium. Eggs will be maintained in 100 mL of the test solution in 250 mL beakers maintained in an incubator acclimated to 20° C.
Section 15.3. Exposure Verification: Samples of test and reference solutions will be analyzed for SP or AC content. These measurements will be taken after 72 hrs and upon termination of the exposure. Samples from each beaker containing SP will be measured for perchlorate amounts according to the guidelines set forth in SOPs **AC-2-11** and **AC-1-01.** Samples from each beaker containing AC will be measured for ammonium amounts according to the guidelines set forth in SOP **IN-4-12.** At the end of the study remaining tadpoles will be euthanized and stored in 10% neutral buffered formalin.

Change in Document: Section 12. TEST ANIMALS: Number: Approximately 7,854.
Section 14. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL: *X. laevis* eggs (N=51) will be exposed to one of ten concentrations of SP and ten concentrations of AC both in duplicate. Two additional beakers will contain eggs (N=51) exposed to FETAX medium. Exposures will be terminated after five days. This will give 102 eggs/larvae per treatment for each of four trials, for a study total of 7,854 eggs/larvae.
Section 15.3. Route/Method of Application: Eggs and larvae will be exposed to SP or AC in the beaker medium. Eggs will be maintained in 100 mL of the test solution in 250 mL beakers maintained at 20 ± 2 °C.

* Sequentially numbered in order of the date that the change is effective

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Change In Study Documentation Form

Section 15.3. Exposure Verification: Samples of test and reference solutions will be analyzed for SP or AC content. These samples will be collected prior to exposure, after the 72 hr solution change, and upon termination of the exposure. Samples from each beaker containing SP will be measured for perchlorate amounts according to the guidelines set forth in SOPs **AC-2-11** and **AC-1-01.** Samples from each beaker containing AC will be measured for ammonium amounts according to the guidelines set forth in SOP **IN-4-12.** At the end of the study remaining tadpoles will be euthanized and frozen for possible contaminant analysis at a later date.

Justification and Impact on Study: Section 12. The experiment will be repeated for a total of four trials to validate the results, increasing the total number of eggs/larvae used.
Section 14. The experiment will be repeated for a total of four trials to validate the results, increasing the total number of eggs/larvae used.
Section 15.3. The use of an incubator is not necessary. Eggs and larvae will be maintained in a temperature and photoperiod controlled room.
Section 15.3. To ensure that the larvae are exposed to the correct solution concentrations, all solutions will be sampled for analysis prior to placement of larvae. Larvae remaining at the end of the study will be frozen for possible perchlorate analysis as originally planned.

Submitted by: Signature: Wanda L. Coleman Date: 11/19/01

Authorized by: Study Director: Wanda L. Coleman Date: 11/19/01

Received by: Quality Assurance Unit: Brian Bridwell Date: 3/19/02

* Sequentially numbered in order of the date that the change is effective

A FINAL REPORT

ENTITLED

SUBLETHAL EFFECTS OF AMMONIUM CHLORIDE, AMMONIUM PERCHLORATE AND SODIUM PERCHLORATE ON THE DEVELOPMENT AND METAMORPHOSIS OF *XENOPUS LAEVIS*

STUDY NUMBER:	XEN-01-03
SPONSOR:	Strategic Environmental Research And Development Program (SERDP) 1155 Herndon Parkway, Suite 900 Herndon, Virginia 20170
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University Texas Tech University Health Sciences Center Box 41163 Lubbock, TX 79409-1163
TEST SITE:	Department of Biological Sciences –**AP** Texas Tech University Box 4-3131 Lubbock, Texas 79409-3131
ANALYTICAL TEST SITE:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Center Box 41163 Lubbock, Texas 79409-1163
RESEARCH INITIATION:	August 29, 2001
RESEARCH COMPLETION:	November 7, 2001

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List of Tables and Figures

GOOD LABORATORIES PRACTICES STATEMENT

Project XEN-01-03, entitled " Sublethal effects of ammonium chloride, ammonium perchlorate, and sodium perchlorate on the development and metamorphosis of *Xenopus laevis*", was performed whenever possible in the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Noted exceptions are as follows:

Submitted By:

James A. Carr, Ph.D

March 28, 2002
Date

QUALITY ASSURANCE STATEMENT

This study was conducted under the Institute of Environmental and Human Health's Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Any changes in protocol and SOPs were documented in writing and signed by the study director.

The Quality Assurance Officer verbally notified the Study Director of all findings at the time of the inspection. Written audit reports were also submitted to the Study Director and Test Facility Management. Audits were performed for the following phases of the project:

Auditable Research Phase / Activity	Audit Dates		Date written report submitted to Study Director	Date written report submitted to Management
	Start	End		
Test Material Mixing	08/29/01	08/29/01	09/04/01	
Final Report and Raw Data Review	03/04/02	03/20/02		

Submitted By:

Ryan Bounds
Quality Assurance Manager

03/28/02
Date

1.0 DESCRIPTIVE STUDY TITLE:
Sublethal effects of ammonium chloride, ammonium perchlorate, and sodium perchlorate on the development and metamorphosis of *Xenopus laevis*.

2.0 STUDY NUMBER: XEN-01-03

3.0 SPONSOR:
Strategic Environmental Research
And Development Program (SERDP)
1155 Herndon Parkway, Suite 900
Herndon, Virginia 20170

4.0 TESTING FACILITY NAME & ADDRESS:
The Institute of Environmental and Human Health
Texas Tech University
Texas Tech University Health Sciences Center
Box 41163
Lubbock, TX 79409-1163

5.0 EXPERIMENTAL START & TERMINATION DATES:
Start Date: August 29, 2001
Termination Date: November 7, 2001

6.0 KEY PERSONNEL:
James A. Carr, Co-Principal Investigator
Wanda L. Goleman, Study Director
Todd Anderson, Analytical Chemist
Ryan Bounds, Quality Assurance Officer
Ron Kendall, Principal Investigator/ Testing Facility Manager

7.0 STUDY SUMMARY

Larval *Xenopus laevis* were exposed to ammonium perchlorate (AP, 38 ppb, 14,040 ppb), sodium perchlorate (SP, 38 ppb, 14,040 ppb), ammonium chloride (AC, 38 ppb, 14,040 ppb) or untreated FETAX medium for 70 d beginning < 24 hr after fertilization to determine the contribution of ammonium ions to the developmental effects of ammonium perchlorate. There were no treatment-related effects on mortality or abnormal swimming. Ammonium chloride at 14,040 ppb reduced snout-vent length. AP and SP, but not AC, completely inhibited metamorphosis at the 14,040 ppb concentration as evidence by reduction in incidence of forelimb emergence, reduction in hindlimb growth, and failure to reabsorb the tail. These data suggest that ammonium ions do not contribute to the effects of AP on thyroid hormone-sensitive indices of development in *X. laevis*.

8.0 STUDY OBJECTIVES / PURPOSE:

To determine the sublethal effects of sodium perchlorate (SP), ammonium perchlorate (AP), and ammonium chloride (AC) on the development and metamorphosis of *Xenopus laevis* when exposed for 70 d, beginning within 24 hr of oviposition.

9.0 TEST MATERIALS:

Test Chemical name: Sodium Perchlorate
CAS number: 7601-89-0
Characterization: 98% (Assay)
Source: Aldrich Chemical Company

Test Chemical name: Ammonium Chloride
CAS number: 12125-02-9
Characterization: 99.99% (Assay)
Source: Aldrich Chemical Company

Test Chemical name: Ammonium Perchlorate
CAS number: 7790-98-9
Characterization: 99.999% pure, found to be stable in reverse osmosis water for 109 days.
Source: Sigma-Aldrich Chemical Company

Reference Chemical name: de-ionized water
CAS number: Not Applicable
Characterization: FETAX (Frog Embryo Teratogenesis Assay – *Xenopus*) medium, a mixture of reagent grade salts, prepared in 100% ultrapure water. The quality of the water will be confirmed by analytical tests.
Source: City tap water, which has been run through reverse osmosis and a de-ionozer to convert it to ultrapure water and contains reagent grade salts according to SOP AQ-1-13 (Sunderman et al., 1991).

10.0 JUSTIFICATION OF TEST SYSTEM

Ionic perchlorate alters calcium balance in fishes and amphibians (Luttgau et al., 1983; Thevenod et al., 1992; Jong et al., 1997) as well as other vertebrates. Calcium is an ubiquitous chemical messenger that is involved in the regulation of cellular function. Endocrine glands require calcium for the normal secretion of hormones and therefore contaminant-induced disruption of calcium balance can lead to systemic endocrine disruption. Perchlorate is also known to prevent intake of iodine from water or food and thus it is goitrogenic (thyroid gland inhibitor) in many animals including fishes and amphibians (Miranda et al., 1996; Manzon and Youson, 1997). Because of the important role played by hormones in development and reproduction, endocrine disruption is likely to lead to serious impairments in growth, reproductive fitness, and consequently, amphibian and wildlife stability as well as human health. Although sublethal effects data already exists for AP, the relative contribution of ammonium to the effects of AP has not

been tested. We will test this possibility directly by examining the effects of SP and AC on development and metamorphosis.

X. laevis are a widely used animal model in basic toxicological, developmental, and reproductive research. Also, there is a considerable database already available for this species. They represent a vertebrate class, amphibians, with distinct developmental and physiological adaptations to their environment. They are also easily and economically maintained and bred in the laboratory. Therefore, this species is ideally suited to examine the sublethal effects of contaminants such as AP on aquatic fauna.

11.0 TEST ANIMALS (Where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test system):
Species: South African Clawed Frog, *Xenopus laevis*
Strain: wild type
Age: eggs, larvae, young adults
Number: approximately 700 eggs/larvae
Source: Laboratory colony and *Xenopus* Express (Homosassa, FL).

12.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Each container was labeled as indicated in SOP AQ-1-17, which includes genus and species name; common name; project name, number, and start date; and the name of the person responsible for animal care. Each container was labeled including the sex of the individuals (if appropriate), date eggs were laid/hatched (if applicable), the name of the test substance and its concentration amount, and date of initial exposure.

13.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

X. laevis eggs (N=50) were exposed to one of 2 concentrations of SP, 2 concentrations of AP, and 2 concentrations of AC, all in duplicate. Two additional containers held eggs (N=50) exposed to FETAX medium alone. Exposures were terminated after 70 d. This gave 100 eggs/larvae per treatment, for a study total of 700 eggs/larvae.

14.0 METHODS:

14.1 Test System Acquisition, Quarantine, Acclimation

Five adult male and five adult female *X. laevis* were obtained from *Xenopus* Express (Homosassa, FL). Refer to SOP AQ-1-06 for details on routine *X. laevis* husbandry. They were maintained in 45-L glass tanks containing 18 L of ultrapure water for 1-2 days at 20 ϴC on a 12L: 12D light regimen. Male and female *X. laevis* were maintained separately for 7 d before breeding. Please refer to SOP AQ-1-04 for details on *X. laevis* breeding.

14.2 Test Condition Establishment

Naturally fertilized eggs were used. They were obtained from five pairs of adults who had been artificially induced to spawn (see *X. laevis* husbandry SOP AQ-1-04). These eggs were collected and examined under a microscope for viability (SOP AQ-1-17). A group of 10 randomly chosen eggs from each of 5 females was added to each

beaker with test concentrations of SP, AP, AC, or FETAX medium. Eggs and tadpoles up to five days of age were held in 250 mL glass beakers containing 100 mL of either test concentrations or control solution. After 5 d, the tadpoles were moved to 21-L glass tanks where they remained throughout the rest of the experiment. Unfertilized eggs were disposed of appropriately. Any undeveloped eggs through the exposure period were noted. Each beaker/tank was labeled as indicated in SOP AQ-1-17, which includes genus and species name, common name, project name, number and start date, sex of the individuals (if appropriate), date eggs were laid/hatched (if applicable), the name of the test substance and its concentration amount, date of initial exposure, and the name of the person responsible for animal care.

14.2a Adults were induced to spawn according to SOP AQ-1-04.

14.2b Groups of 50 eggs, representing 10 randomly chosen eggs from each of 5 females, were placed into appropriately labeled beakers containing a single concentration of test solution in FETAX medium or FETAX medium alone.

14.3 Test Material Application

Test material was premixed to appropriate concentrations and added to the appropriately labeled glass aquaria (see section 15.2). Larvae were added to 21 L glass aquaria containing 8 L of test or reference solution. A 50% solution change containing the identical concentration of test substance was performed daily.

Rates/concentrations: 0, 38 ppb, 14040 ppb.

Frequency: Constant Exposure for 70-d.

Route/Method of Application: Eggs and larvae were exposed to SP, AP, AC, or FETAX in the beaker medium. Eggs were maintained in 100 mL of the test solution in 250 mL beakers maintained in a water bath acclimated to 20 $\pm$ 2 ϴC for the first 5-d. After five days, the animals were transferred into 21-L glass tanks filled with 8 L of the appropriate test solution. Test and reference solutions were changed every 72 hrs. Medium containing the identical concentration of test substance was added back to each beaker as needed to maintain test conditions. The stock solutions were made in 100 fold concentrations (Table 1), stored in 1 L amber bottles, and added to the appropriate containers according to Table 2.

Table 1. Preparation of Test Solutions and Initial Solutions

Chemical	Initial Stock	Dilution Factor	Final Concentration (ppb*)
AP	1.404 g/L	1:100	14040
AP	3.8 mg/L	1:100	38
SP	1.404 g/L	1:100	14040
SP	3.8 mg/L	1:100	38
AC	1.404 g/L	1:100	14040
AC	3.8 mg/L	1:100	38

*ppb = Γg/L

Table 2. Addition to the Tanks During a 50% Change

	38 ppb* 250 mL beaker	14040 ppb 250 mL beaker	38 ppb 21-L tank	14040 ppb 21-L tank	FETAX 21-L tank
Ultra Pure Water	44.5 mL	44.5 mL	3560 mL	3560 mL	3600 mL
10 X FETAX	5 mL	5 mL	400 mL	400 mL	400 mL
100 X (3.8 mg/L)	0.5 mL	0.0 mL	40 mL	0 mL	0 mL
100 X (1.404 g/L)	0.0 mL	0.5 mL	0 mL	40 mL	0 mL

*ppb = Γg/L

Method of application was immersion. Route of exposure was via dermal, oral, and respiratory exposure as the chemical was in the beaker/aquaria medium.

Justification for Exposure Route: *X. laevis* are fully aquatic as larvae and as adults.

Exposure Verification: Samples of stock and reference solutions were collected for perchlorate or ammonium content prior to initiation of the experiment. Additional samples from premixed test solutions were taken every 72 hr. Samples were also collected from the tank solutions approximately mid-way through the study and at completion of the exposure period.

14.4 Test System Observation

Beginning on the day of hatch, hatching success (# unhatched eggs/total # eggs), % deformities (# showing bent tails, asymmetric tails/total hatched), edema (% showing distension of body with fluid/total hatched), and abnormal swimming (% showing abnormal swimming/total hatched) were noted daily for each test and reference solution. For free swimming larvae, % mortality (#dead/total hatched), percent showing deformities, percent showing abnormal swimming behavior, percent fore limb emergence (# showing fore limb emergence/ # observed), and percent tail resorption (# animals with completely resorbed tails/ # observed) were recorded. Water quality, including dissolved oxygen, salinity, conductivity, and pH was performed at least once per week. Temperature of a surrogate tank with the same volume of water was noted every day. Dead animals were removed and preserved in 10% neutral buffered formalin (NBF).

14.5 Animal Sacrifice and Sample Collections

The experiment was terminated after 70-d. Remaining tadpoles were immersed in MS-222 (3-aminobenzoic acid ethyl ester, 0.1% solution) according to SOP AQ-1-03.

Five larvae per tank were frozen for perchlorate analysis. Remaining larvae were preserved in 10% NBF.

14.6 Endpoint Analysis

Hatching success, deformities (bent tails, asymmetric tails), edema (distension of body with fluid), and abnormal swimming were noted for hatchlings. Nieuwkoop and Faber (1967) stage, snout-vent length, hind limb length (SOP AQ-1-12), deformities, and abnormal swimming behavior were recorded for larvae.

15.0 STATISTICAL METHODS

Snout-vent length, hind limb length, and tail length were analyzed by one-way ANOVA.

16.0 PROTOCOL CHANGES / REVISIONS:

See attached change in study documentation forms.

17.0 RESULTS:

There were no treatment related effects on hatching success. Hatching success ranged from 76-78% in FETAX controls, 84-88% in 38 ppb AP, 82-86% in 14,040 ppb AP, 76-80% in 38 ppb SP, 82% in 14,040 ppb SP, 84-87% in 38 ppb AC, and 75-76% in 14,040 ppb AC. Post-hatch survival also was not affected by treatment condition, although there were tank-related effects on post-hatch survival. Post-hatch mortality (68 d) was 8-23% in FETAX controls, 5-17% in 38 ppb AP, 10-35% in 14,040 ppb AP, 8-10% in 38 ppb SP, 8-12% in 14,040 ppb SP, 22% in one 38 ppb AC tank and 60% in a second tank. It is unlikely that the high mortality in one of the 38 ppb AC tanks was treatment related, as in our hands the LC_{50} for AC in *X. laevis* larvae is 118 ppm. Furthermore, post-hatch mortality ranged from 13-21% in 14,040 ppb AC. There were no treatment-related effects on incidence of edema or asymmetric tails.

Results of the perchlorate analysis reveal that the nominal perchlorate dosing solutions were close to their desired target concentrations, (Table 3), whereas the AC and FETAX dosing solution contained only trace amounts of perchlorate. There were no noticeable treatment effects on abnormal swimming. The incidence of abnormal swimming ranged from 3–8% in FETAX controls, 0-7% in 38 ppb AP, 0-14% in 14,040 ppb AP, 0-3 % in 38 ppb SP, 0-3% in 14,040 SP, 2-9% in 38 ppb AC, and 0-8% in 14,040 ppb AC. There were treatment related effects on the incidence of forelimb emergence (FLE). Percent FLE in FETAX animals averaged 61%. Mean percent FLE was slightly reduced in the 38 ppb AP treatment group (45.5%) but not the 38 ppb SP or AC treatments (67% and 51%, respectively). Exposure to 14,040 ppb AP or SP reduced % FLE to zero, whereas the incidence of FLE in the 14,040 ppb AC treatment group was 47 %.

Treatment effects on snout-vent length (an indicator of somatic growth, SVL), tail length (an indicator of tail resorption during metamorphosis), and hindlimb length (an indicator of thyroid-dependent somatic growth, HLL, Goleman et al., 2002a) are shown in Figs. 1-3. None of the treatments affected SVL except the 14,040 concentration of AC, which reduced SVL slightly relative to controls. Tail length and HLL were affected in a predictable manner by AP and SP. Tail length in the 14,040 ppb AP and SP treatment groups was significantly greater than FETAX controls because none of the animals in these treatments completed metamorphosis. Tail length in the 14,040 ppb AC treatment group was statistically no different than the FETAX controls. Likewise, hindlimb growth was reduced in the 14,040 ppb AP and SP treatment groups compared to the FETAX controls but was unaffected by the same concentration of AC (Fig. 3).

Table 3. Perchlorate Content of Dosing Solutions[a].

Treatment		Nominal perchlorate (ppb)	Actual perchlorate (ppb)
Ammonium Perchlorate			
	38	38	36.7 ± 1.34
	14,040	14,040	12,507 ± 539
Sodium Perchlorate			
	38	38	38.8 ± 1.70
	14,040	14,040	11,918 ± 483
Ammonium Chloride			
	38	0	0.00 ± 0.00
	14,040	0	9.91 ± 9.91
FETAX			0.09 ± 0.09

[a]From aliquots of diluted stock solutions prepared every 3 d from 8/29/01 to 11/05/01.

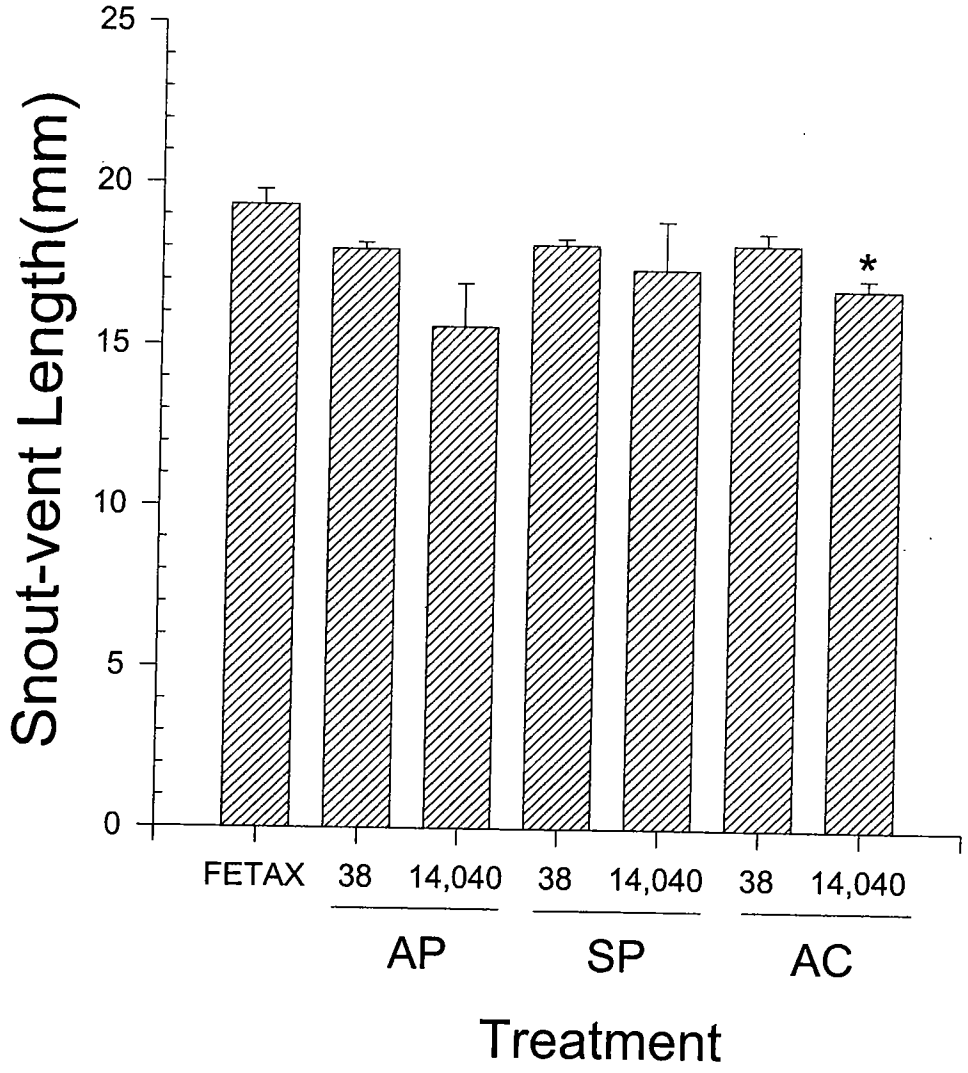

Figure 1. Influence of perchlorate salts and ammonium chloride on snout-vent length after a 70-d exposure beginning < 24 hr after fertilization. Bars represent the mean + SE of 10-46 animals per group from two replicates.
* Significantly reduced relative to control.

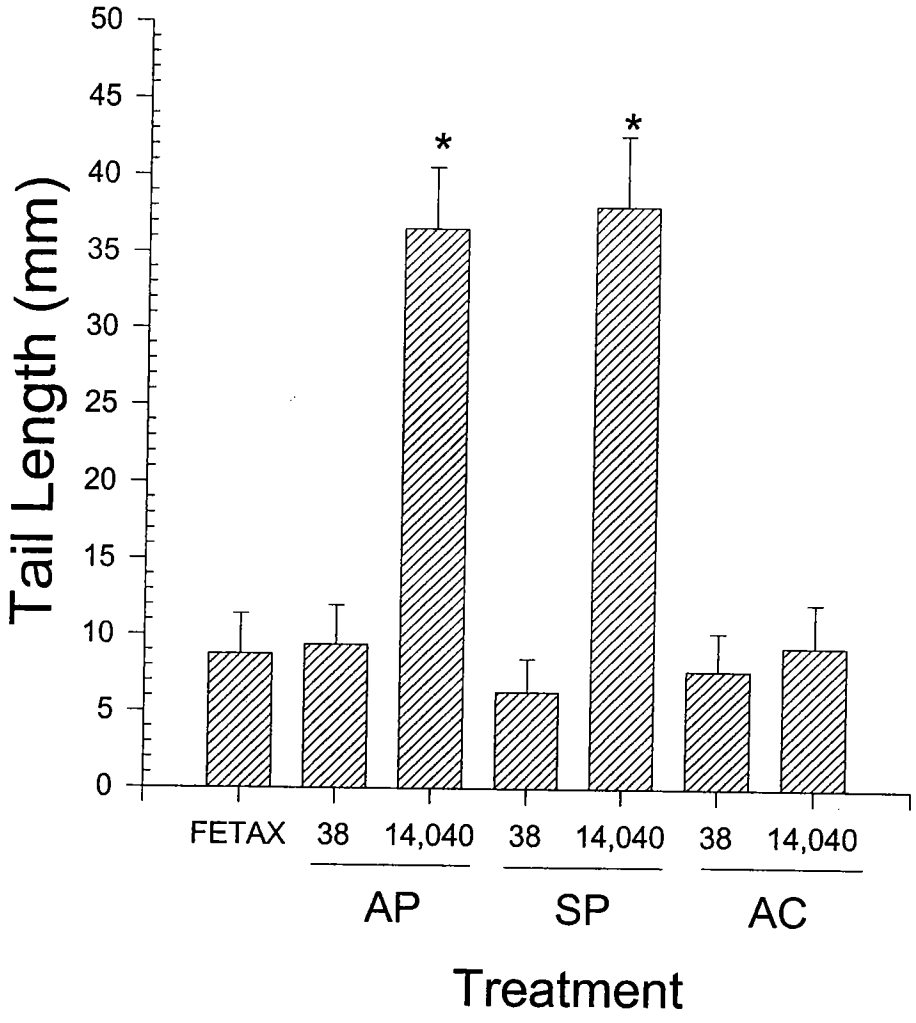

Figure 2. Influence of perchlorate salts and ammonium chloride on tail length after a 70-d exposure beginning < 24 hr after fertilization. Bars represent the mean + SE of 10-46 animals per group from two replicates.
* Significantly greater relative to control.

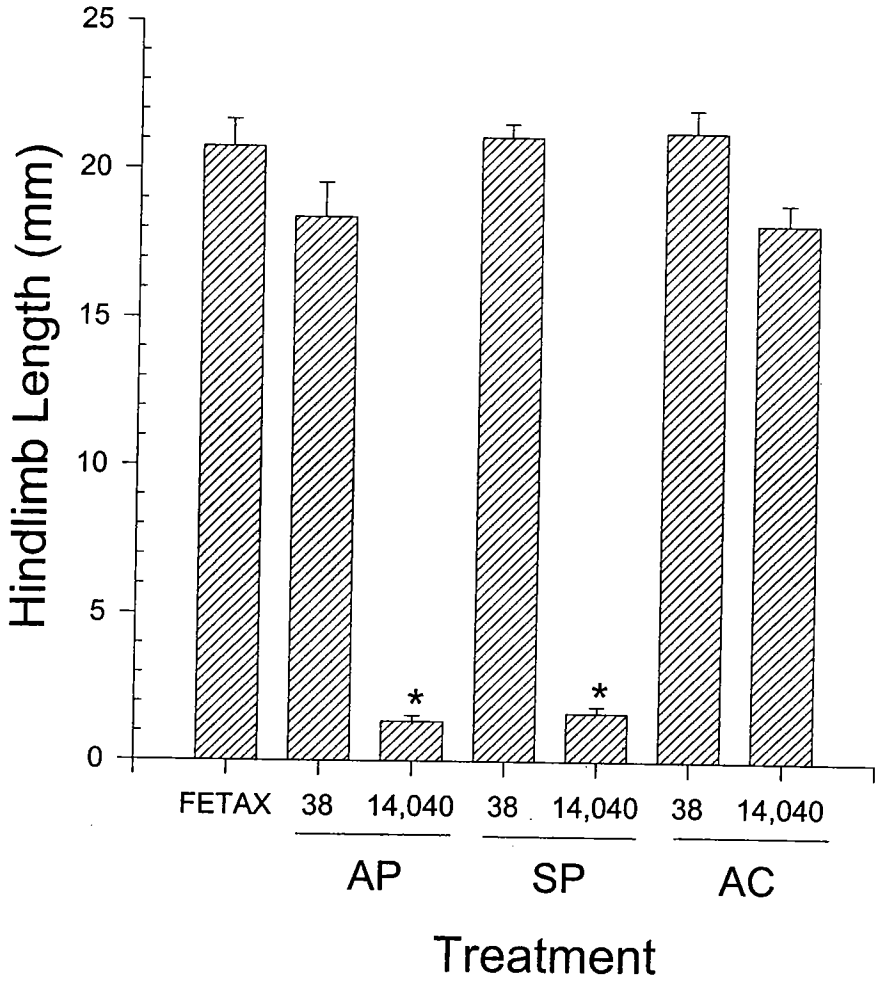

Figure 3. Influence of perchlorate salts and ammonium chloride on hindlimb length after a 70-d exposure beginning < 24 hr after fertilization. Bars represent the mean + SE of 10-46 animals per group from two replicates.
* Significantly reduced relative to control.

18.0 DISCUSSION

None of the treatments affected hatching success or post-hatch mortality. Hatching success was high, averaging greater than 75% in all groups, suggesting that healthy embryos were employed in this study. The concentrations of AP, SP, and AC employed in the present study are well below the LC_{50}s for these compounds (Goleman et al., 2002; Carr, XEN-01-01 final report).

A noteworthy finding of the present study is the lack of AC effects on endpoints of metamorphosis. Both SP and AP at the 14 ppm concentration completely prevented metamorphosis as determined by reduced hindlimb growth, absence of forelimb emergence, and failure to initiate tail resportion in these groups. The same concentration of AC had no effect on any of these parameters. To the extent that all of these parameters are thyroid-sensitive indicators of metamorphosis, are data indicate clearly that ammonium ions do not contribute to thyroid disruption observed with AP.

19.0 STUDY RECORDS AND ARCHIVE:

Study Records will be maintained at The Institute of Environmental and Human Health (TIEHH) Archive for a minimum of one year after study completion date.

20.0 REFERENCES:

Anderson, T.A., and T. H. Wu. (2002). Extraction, cleanup, and analysis of the perchlorate anion in tissue samples. Bull Environ. Contam. Toxicol. In press.

Goleman,W.L., Urquidi, L.J., Anderson, T.A., Kendall, R.J., Smith E.E., and Carr, J.A. (2002). Environmentally relevant concentrations of ammonium perchlorate inhibit development and metamorphosis in *Xenopus laevis*. Environ. Toxicol. Chem. 21: 424-430.

Manzon, R.G. and Youson, J.H. (1997). The effects of exogenous thyroxine (T_4) on triiodothyronine (T_3), in the presence or absence of potassium perchlorate, on the incidence of metamorphosis and on serum T_4 and T_3 concentrations in larval sea lampreys (*Petromyzon marinus* L). Gen. Comp. Endocrinol. 106: 211-220.

Miranda, L.A., Pisano, A. and Casco, V. (1996). Ultrastructural study of thyroid glands of *Bufo arenarum* larvae kept in potassium perchlorate solution. Biocell 20: 147-153.

Siglin, J.C., Mattie, D.R., Dodd, D.E., Hildebrandt, P.K., Baker, W.H. (2000). A 90-day drinking water toxicity study in rats of the environmental contaminant ammonium perchlorate. Toxicol. Sci. 57:61-74.

Taylor, A.C. and Kollros, J..J. (1946). Stages in the normal development of *Rana pipens* larvae. Anat. Rec. 94: 7-23.

Urbansky, E.T. (1998). Perchlorate chemistry: implications for analysis and remediation. Bioremediation J. 2: 81-95.

21.0 APPENDICES:

Study Protocol
Changes to Study Documentation

Project No. T9700.2

A STUDY PROTOCOL

ENTITLED

SUBLETHAL EFFECTS OF AMMONIUM CHLORIDE, AMMONIUM PERCHLORATE AND SODIUM PERCHLORATE ON THE DEVELOPMENT AND METAMORPHOSIS OF *XENOPUS LAEVIS*

STUDY/PROTOCOL NUMBER: XEN-01-03

SPONSOR:

United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

TESTING FACILITY:

Name/Address: The Institute of Environmental and Human Health
Texas Tech University
Texas Tech University Health Sciences Center
Box 41163
Lubbock, TX 79409-1163

Test Facility Management: Dr. Ronald J. Kendall

Study Director: Wanda Goleman

PROPOSED EXPERIMENTAL
START DATE: JULY 1, 2001

1. **DESCRIPTIVE STUDY TITLE:** Sublethal effects of ammonium chloride, ammonium perchlorate, and sodium perchlorate on the development and metamorphosis of *Xenopus laevis*.

2. **STUDY NUMBER: XEN-01-03**

3. **SPONSOR:** United States Air Force
IERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

4. **TESTING FACILITY NAME & ADDRESS:**
The Institute of Environmental and Human Health
Texas Tech University
Texas Tech University Health Sciences Center
Box 41163
Lubbock, TX 79409-1163

5. **PROPOSED EXPERIMENTAL START & TERMINATION DATES:**
Start Date: July 1, 2001
Termination Date: September 15, 2001

6. **KEY PERSONNEL:**
James A. Carr, Co-Principle Investigator
Wanda Goleman, Study Director
John Blevins, Graduate Research Assistant
Todd Anderson, Analytical Chemist
Ryan Bounds, Quality Assurance Manager
Ken Dixon, Statistical/Modeling Support
Ron Kendall, Principle Investigator / Testing Facility Management

7. DATED SIGNATURES:

_____	_____	Wanda Goleman Study Director
_____	_____	Dr. James Carr Co-Principle Investigator
_____	_____	Dr. Todd Anderson Analytical Chemist
_____	_____	Ryan Bounds Quality Assurance Manager
_____	_____	Dr. Ken Dixon Statistician
_____	_____	Dr. Ron Kendall Principle Investigator/ Testing Facility Management

8. REGULATORY COMPLIANCE STATEMENT

Quality Control and Quality Assurance

This study will be conducted in accordance with established Quality Assurance program guidelines and in compliance, where appropriate and possible, with Good Laboratory Practice Standards (40 CFR Part 160, August 17, 1989).

Document Control Statement

This document is considered proprietary to and the Sponsor. Do not copy, quote or distribute. For access to this document or authority to release or distribute, please write to:

Dr. James Carr
Department of Biological Sciences
Texas Tech University
Box 4-3131
Lubbock, Texas 79409

9. STUDY OBJECTIVES / PURPOSE:

To determine the sublethal effects of sodium perchlorate (SP), ammonium perchlorate (AP), and ammonium chloride (AC) on the development and metamorphosis of *Xenopus laevis* when exposed for 70 days, beginning within 24 hours of oviposition.

10. TEST MATERIALS:

Test Chemical name: Sodium Perchlorate
CAS number: 7601-89-0
Characterization: 98% (Assay)
Source: Aldrich Chemical Company

Test Chemical name: Ammonium Chloride
CAS number: 12125-02-9
Characterization: 99.99% (Assay)
Source: Aldrich Chemical Company

Test Chemical name: Ammonium Perchlorate
CAS number: 7790-98-9
Characterization: 99.999% pure, found to be stable in reverse osmosis water for 109 days.
Source: Sigma-Aldrich Chemical Company

Reference Chemical name: de-ionized water
CAS number: Not Applicable
Characterization: FETAX (Frog Embryo Teratogenesis Assay – *Xenopus*) medium, a mixture of reagent grade salts, prepared in 100% ultrapure water. The quality of the water will be confirmed by analytical tests.
Source: City tap water, which has been run through reverse osmosis and a de-ionozer to convert it to ultrapure water and contains reagent grade salts according to **SOP AQ-1-13** (Sunderman et al., 1991).

11. JUSTIFICATION OF TEST SYSTEM

Ionic perchlorate alters calcium balance in fishes and amphibians (Luttgau et al., 1983; Thevenod et al., 1992; Jong et al., 1997) as well as other vertebrates. Calcium is an ubiquitous chemical messenger that is involved in the regulation of cellular function. Endocrine glands require calcium for the normal secretion of hormones and therefore contaminant-induced disruption of calcium balance can lead to systemic endocrine disruption. Perchlorate is also known to prevent intake of iodine from water or food and thus it is goitrogenic (thyroid gland inhibitor) in many animals including fishes and amphibians (Miranda et al., 1996; Manzon and Youson, 1997). Because of the important role played by hormones in development and reproduction, endocrine disruption is likely to lead to serious impairments in growth, reproductive fitness, and consequently,

amphibian and wildlife stability as well as human health. Although sublethal effects data already exists for AP, the relative contribution of ammonium to the effects of AP has not been tested. We will test this possibility directly by examining the effects of SP and AC on development and metamorphosis.

X. laevis are a widely used animal model in basic toxicological, developmental, and reproductive research. Also, there is a considerable database already available for this species. They represent a vertebrate class, amphibians, with distinct developmental and physiological adaptations to their environment. They are also easily and economically maintained and bred in the laboratory. Therefore, this species is ideally suited to examine the sublethal effects of contaminants such as AP on aquatic fauna.

12. **TEST ANIMALS :**

Species: South African Clawed Frog, *Xenopus laevis*
Strain: wild type
Age: eggs, larvae, young adults
Number: approximately 714 eggs/larvae
Source: Laboratory colony and *Xenopus* Express (Homosassa, FL)

13. **PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:**

Each container will be labeled as indicated in **SOP AQ-1-17**, which includes genus and species name; common name; project name, number, and start date; and the name of the person responsible for animal care. Each container will be labeled including the sex of the individuals (if appropriate), date eggs were laid/hatched (if applicable), the name of the test substance and its concentration amount, and date of initial exposure.

14. **EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:**

X. laevis eggs (N=51) will be exposed to one of 2 concentrations of SP, 2 concentrations of AC, and 2 concentrations of AC all in duplicate. Two additional containers will contain eggs (N=51) exposed to FETAX medium alone. Exposures will be terminated after 70 d. This will give 102 eggs/larvae per treatment, for a study total of 714 eggs/larvae.

15. **METHODS:**

15.1 **Test System acquisition, quarantine, acclimation**

Three adult male and three adult female *X. laevis* will be obtained from *Xenopus* Express (Homosassa, FL). Refer to **SOP AQ-1-06** for details on routine *X. laevis* husbandry. They will be maintained in 45-L glass tanks containing 18 L of ultrapure water for 1-2 days at 20 C on a 12L: 12D light regimen. Male and female *X. laevis* will be maintained separately for 7 d before breeding. Please refer to **SOP AQ-1-04** for details on *X. laevis* breeding.

15.2 Test condition establishment

Naturally fertilized eggs will be used. They will be obtained from three pairs of adults who have been artificially induced to spawn (see *X. laevis* husbandry **SOP AQ-1-04**). These eggs will be collected and examined under a microscope for viability (**SOP AQ-1-17**). A group of 17 randomly chosen eggs from each of 3 females, for a total of 51 eggs per container, will be added to each beaker with test concentrations of AC, AP, SP, or FETAX medium. Eggs and tadpoles up to five days of age will be held in 250 mL glass beakers containing either test concentrations or control solution. After 5 days the tadpoles will be moved to 20L tanks where they will remain throughout the rest of the experiment. Unfertilized eggs will be disposed of appropriately. Any undeveloped eggs through the exposure period will be noted. Each beaker/tank will be labeled as indicated in **SOP AQ-1-17**, which includes genus and species name, common name, project name, number and start date, sex of the individuals (if appropriate), date eggs were laid/hatched (if applicable), the name of the test substance and its concentration amount, date of initial exposure, and the name of the person responsible for animal care.

15.2a Adults will be induced to spawn according to **SOP AQ-1-04**.

15.2b Groups of 51 eggs, representing 17 randomly chosen eggs from each of 3 females, will be placed into appropriately labeled beakers containing a single concentration of test solution in FETAX medium or FETAX medium alone.

15.3 Test Material Application

Rates/concentrations: 38 ppb, 14040 ppb.

Frequency: Constant Exposure for 70-d.

Route/Method of Application: Eggs and larvae will be exposed to SP, AC, AP, or FETAX in the beaker medium. Eggs will be maintained in 100 mL of the test solution in 250 mL beakers maintained in an incubator acclimated to 20 C for the first 5-d. After five days, the animals will be transferred into 20 L tanks filled with 8 L of the appropriate test solution. Test and reference solutions will be changed every 72 hrs. Medium containing the identical concentration of test substance will be added back to each beaker as needed to maintain test conditions. The stock solutions will be made in 100 fold concentrations, stored in 1 L amber bottles, and added according to the chart below.

Addition to the Tanks After a 50% Change

	38 ppb	14040 ppb	FETAX	250 mL beaker 38 ppb	250 mL beaker 14040 ppb
Ultra Pure Water	3560 mL	3560 mL	3600 mL	44.5 mL	44.5 mL
10 X FETAX	400 mL	400 mL	400 mL	5 mL	5 mL
100 X (3.8 mg/L)	40 mL	0 mL	0 mL	0.5 mL	0.0 mL
100 X (1.404 g/L)	0 mL	40 mL	0 mL	0.0 mL	0.5 mL

Preparation of Test Solutions

Chemical	Initial Stock	Dilution Factor	Final Concentration (µg/L)	Final Concentration (ppb)
AP	1.404 g/L	1:100	14040	14040
AP	3.8 mg/L	1:100	38	38
SP	1.404 g/L	1:100	14040	14040
SP	3.8 mg/L	1:100	38	38
AC	1.404 g/L	1:100	14040	14040
AC	3.8 mg/L	1:100	38	38

Method of application will be immersion. Route of exposure will be via dermal, oral, and respiratory exposure as the chemical will be in the beaker/aquaria medium.

Justification for Exposure Route: *X. laevis* are fully aquatic as larvae and as adults.

Exposure Verification: Samples of stock and reference solutions will be collected for perchlorate or ammonium content prior to initiation of the experiment. Additional samples from test solutions will be taken every 72 hrs.

15.4 Test System Observation

Beginning on the day of hatch, hatching success (# unhatched eggs/Total # eggs), % deformities (# showing bent tails, asymmetric tails/total hatched), edema (% showing distension of body with fluid/total hatched), and abnormal swimming (% showing abnormal swimming/total) will be noted daily for each test and reference solution. For free swimming larvae, % mortality (#dead/#hatched), percent showing deformities,

percent showing abnormal swimming behavior, percent front leg emergence (# showing fore limb emergence/ # observed), percent tail resorption (# animals with completely resorbed tails), temperature of a surrogate tank with the same volume of water will be noted every day. Water quality such dissolved oxygen, salinity, and conductivity will be taken once per week. Dead animals will be removed and preserved in 10% neutral buffered formalin (NBF).

15.5 Animal Sacrifice and Sample Collections

The experiment will be terminated after 70-d. Remaining tadpoles will be immersed in MS-222 (3-aminobenzoic acid ethyl ester, 0.1% solution) according to **SOP AQ-1-03** then preserved in 10% NBF.

15.6 Endpoint Analysis

Hatching success, deformities (bent tails, asymmetric tails), edema (distension of body with fluid), and abnormal swimming will be noted for hatchlings. Nieuwkoop and Faber (1967) stage, total body length, hind limb length (**SOP AQ-1-12**), deformities, and abnormal swimming behavior will be recorded for larvae.

16. PROPOSED STATISTICAL METHODS

Probit analysis will be used to determine the effects of the sublethal concentration percentages.

17. REPORT CONTENT/RECORDS TO BE MAINTAINED:

Records to be maintained include.

- Incubator and water temperature, salinity, pH, dissolved oxygen, ammonia, and conductivity will be collected
- Date, time and amount of feedings per tank/beaker will be recorded. Number of expired larvae removed prior to termination of exposure will be recorded including each date and beaker.
- Deformities and abnormal swimming behavior will be recorded daily prior to termination of the experiment.
- Fore limb emergence and tail resorption will be recorded daily prior to termination of the experiment.

Report content will also include presentation of data, interpretation, and discussion of the following endpoints:

List individual endpoints and analyses.
Discussion of the relevance of findings
List of all SOPs used
List of all personnel

18. RECORDS TO BE MAINTAINED / LOCATION:

The final report will be delivered to the Sponsor on or before **February 28, 2002**. Copies of all data, documentation, records, protocol information, as well as the specimens shall be sent to the Sponsor, or designated delivery point, upon request (within six months of study completion). All data, the protocol and a copy of the final report shall be maintained by the testing facility.

19. QUALITY ASSURANCE:

The Quality Assurance Unit will inspect the study at intervals to insure the integrity of the study. Written records will be maintained indicating but not limited to the following: date of inspection, study inspected, phase inspected, person conducting the inspection, findings and problems, recommended and taken action, and any scheduled reinspections. Any problems likely to effect study integrity shall be brought to the immediate attention of the Study Director. The Quality Assurance Unit will periodically submit written status reports on the study to management and the Study Director.

20. PROTOCOL CHANGES / REVISIONS:

All changes and/or revisions to the protocol, and the reasons therefore, shall be documented, signed and dated by the Study Director and maintained with the protocol and the Quality Assurance Unit.

21. REFERENCES:

Luttgau, H.C., Gottschalk, G., Kovacs, L., Fuxreiter, M. (1983). How perchlorate improves excitation-contraction coupling in skeletal muscle fibers. Biophys. J. 43:247-249.

Jong, D. S., Stroffekova, K., Heiny, J.A. (1997). A surface potential change in the membranes of frog skeletal muscle is associated with excitation-contraction coupling. J. Physiol. 499:787-808.

Manzon, R.G. and Youson, J.H. (1997). The effects of exogenous thyroxine (T_4) on triiodothyronine (T_3), in the presence or absence of potassium perchlorate, on the incidence of metamorphosis and on serum T_4 and T_3 concentrations in larvae sea lampreys (*Petromyzon marinus* L). Gen. Comp. Endocrinol. 106:211-220.

Miranda, L.A., Pisano, A. and Casco V. (1996). Ultrastructural study of thyroid glands of *Bufo arenarum* larvae kept in potassium perchlorate solution. Biocell 20:147-143.

Nieuwkoop, P.D. and Faber, J. (1967). Normal table of *Xenopus laevis (Daudin)*, North Holland, Amsterdam.

Sunderman, F.W., Plowman, M.C. and Hopfer, S.M. (1991). Embryotoxicity and teratogenicity of cadmium chloride in *Xenopus laevis*, assayed by the FETAX procedure. Ann. Clin. Lab. Sci. 21:381-391.

Thevenod, F. and Jones, S.W. (1992). Cadmium block of calcium current in frog sympathetic neurons. Biophys. J. 63:162-168.

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Form No. 014 Rev. 3.06/00
Project No.: T9700.2
*Change No.: 1
Page: 1 of 1

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: X Amendment ____Deviation ____ Addendums

Document Reference Information

Check One: X Protocol ____ SOP ____ Other _____

Title: Sublethal effects of ammonium chloride, ammonium perchlorate and sodium perchlorate on the development and metamorphosis of *Xenopus laevis*
Dated: July 1, 2001
Document # (if appropriate): XEN-01-03 protocol
Page #(s): 6, 8
Section #: 15.1, 17
Text to reference: 15.1 Test Material Application. Route/Method of Application: Eggs and larvae will be exposed to SP, AP, AC, or FETAX in the beaker medium. Eggs will be maintained in 100 mL of the test solution in 250 mL beakers maintained in an incubator acclimated to 20 °C for the first 5-d. After five days, the animals will be transferred into 21-L glass tanks filled with 8 L of the appropriate test solution.
17. Report content/records to be maintained: Records to be maintained include.

- Incubator and water temperature, salinity, pH, dissolved oxygen, ammonia, and conductivity will be collected.

Change in Document: 15.1 Test Material Application. Route/Method of Application: Eggs and larvae will be exposed to SP, AP, AC, or FETAX in the beaker medium. Eggs will be maintained in 100 mL of the test solution in 250 mL beakers maintained in a water bath acclimated to 22 ± 2 °C for the first 5-d. After five days, the animals will be transferred into 21-L glass tanks filled with 8 L of the appropriate test solution acclimated to 22 ± 2 °C.
17. Report content/records to be maintained: Records to be maintained include.

- Water temperature, salinity, pH, dissolved oxygen, ammonia, and conductivity will be collected.

Justification and Impact on Study: 15.1 Test Material Application. Route/Method of Application: Maintenance in an incubator is not necessary. Room temperature (TIEHH Rm. No. 123) is maintained at an adequate temperature for the development of *Xenopus laevis* eggs and larvae.
17. Report content/records to be maintained: An incubator will not be used during the experiment.

Submitted by: Signature: Wanda R. Goleman Date: 8/22/01
Authorized by: Study Director: Wanda R. Goleman Date: 8/22/01
Received by: Quality Assurance Unit: _____ Date: 3/19/02

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Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: ____ Amendment __X__ Deviation ____ Addendums

Document Reference Information

Check One: __X__ Protocol ____ SOP ____ Other _____

Title: Sublethal effects of ammonium chloride, ammonium perchlorate and sodium perchlorate on the development and metamorphosis of *Xenopus laevis*
Dated: July 1, 2001
Document # (if appropriate): XEN-01-03 protocol
Page #(s): 5, 6
Section #: 12, 14, 15.1, 15.2
Text to reference: Section 12. TEST ANIMALS: Species: South African Clawed Frog, *Xenopus laevis*, Strain: wild type, Age: eggs, larvae, young adults, Number: approximately 714 eggs/larvae, Source: Laboratory colony and *Xenopus* Express (Homosassa, FL).
Section 14. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL: *X. laevis* eggs (N=51) will be exposed to one of 2 concentrations of SP, 2 concentrations of AP, and 2 concentrations of AC, all in duplicate. Two additional containers will contain eggs (N=51) exposed to FETAX medium alone. Exposures will be terminated after 70 d. This will give 102 eggs/larvae per treatment, for a study total of 714 eggs/larvae.
Section 15.1. Test System acquisition, quarantine, acclimation: Three adult male and three adult female *X. laevis* will be obtained from *Xenopus* Express (Homosassa, FL). Refer to **SOP AQ-1-06** for details on routine *X. laevis* husbandry. They will be maintained in 45-L glass tanks containing 18 L of ultrapure water for 1-2 days at 20 °C on a 12L: 12D light regimen. Male and female *X .laevis* will be maintained separately for 7 d before breeding. Please refer to **SOP AQ-1-04** for details on *X. laevis* breeding.
Section 15.2. Test condition establishment: Naturally fertilized eggs will be used. They will be obtained from three pairs of adults who have been artificially induced to spawn (see *X. laevis* husbandry **SOP AQ-1-04**). These eggs will be collected and examined under a microscope for viability (**SOP AQ-1-17**). A group of 17 randomly chosen eggs from each of 3 females will be added to each beaker with test concentrations of SP, AP, AC, or FETAX medium.
Section 15.2b. Groups of 51 eggs, representing 17 randomly chosen eggs from each of 3 females, will be placed into appropriately labeled beakers containing a single concentration of test solution in FETAX medium or FETAX medium alone.

Change in Document: Section 12. TEST ANIMALS: Species: South African Clawed Frog, *Xenopus laevis*, Strain: wild type, Age: eggs, larvae, young adults, Number: approximately 700 eggs/larvae, Source: Laboratory colony and *Xenopus* Express (Homosassa, FL).
Section 14. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL: *X. laevis* eggs (N=50) will be exposed to one of 2 concentrations of SP, 2 concentrations of AP, and 2 concentrations of AC, all in duplicate. Two additional containers will contain eggs (N=50) exposed to FETAX

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Change In Study Documentation Form

medium alone. Exposures will be terminated after 70 d. This will give 100 eggs/larvae per treatment, for a study total of 700 eggs/larvae.
Section 15.1. Test System acquisition, quarantine, acclimation: Five adult male and five adult female *X. laevis* will be obtained from *Xenopus* Express (Homosassa, FL). Refer to **SOP AQ-1-06** for details on routine *X. laevis* husbandry. They will be maintained in 45-L glass tanks containing 18 L of ultrapure water for 1-2 days at 20 °C on a 12L: 12D light regimen. Male and female *X .laevis* will be maintained separately for 7 d before breeding. Please refer to **SOP AQ-1-04** for details on *X. laevis* breeding.
Section 15.2. Test condition establishment: Naturally fertilized eggs will be used. They will be obtained from five pairs of adults who have been artificially induced to spawn (see *X. laevis* husbandry **SOP AQ-1-04**). These eggs will be collected and examined under a microscope for viability (**SOP AQ-1-17**). A group of 10 randomly chosen eggs from each of 5 females will be added to each beaker with test concentrations of SP, AP, AC, or FETAX medium.
Section 15.2b. Groups of 50 eggs, representing 10 randomly chosen eggs from each of 5 females, will be placed into appropriately labeled beakers containing a single concentration of test solution in FETAX medium or FETAX medium alone.

Justification and Impact on Study: Section 12. Five adult pairs were bred instead of three with only 10 eggs collected from each pair, resulting a reduction of eggs used to 700.
Section 14. Five adult pairs were bred instead of three with only 10 eggs collected from each pair, resulting a reduction of eggs used to 700.
Section 15.1. Five adult pairs were obtained and bred for this study.
Section 15.2. Five adult pairs were bred instead of three with only 10 eggs collected from each pair.
Section 15.2b. Five adult pairs were bred instead of three with 10 eggs collected from each pair for a total of 50 eggs placed into each beaker.

Although there is a slight reduction in the number of eggs/ larvae exposed to each treatment, 100 instead of 102, breeding and collecting eggs from 5 breeding pairs increases the genetic variation of the exposed individuals.

Submitted by: Signature: Wanda L Goleman Date: 8/30/01

Authorized by: Study Director: Wanda L Goleman Date: 8/30/01

Received by: Quality Assurance Unit: [signature] Date: 3/19/02

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Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: X Amendment ____Deviation ____ Addendums

Document Reference Information

Check One: X Protocol ____ SOP ____ Other ____

Title: Sublethal effects of ammonium chloride, ammonium perchlorate and sodium perchlorate on the development and metamorphosis of *Xenopus laevis*
Dated: July 1, 2001
Document # (if appropriate): XEN-01-03 protocol
Page #(s): 8
Section #: 15.6
Text to reference: 15.6 Endpoint Analysis: Hatching success, deformities (bent tails, asymmetric tails), edema (distension of body with fluid), and abnormal swimming will be noted for hatchlings. Nieuwkoop and Faber (1967) stage, total body length, hind limb length (**SOP AQ-1-12**), deformities, and abnormal swimming behavior will be recorded for larvae.

Change in Document: 15.6 Endpoint Analysis: Hatching success, deformities (bent tails, asymmetric tails), edema (distension of body with fluid), and abnormal swimming will be noted for hatchlings. Nieuwkoop and Faber (1967) stage, snout-vent length, hind limb length (**SOP AQ-1-12**), deformities, and abnormal swimming behavior will be recorded for larvae.

Justification and Impact on Study: Snout-vent length is a more appropriate measurement for larvae of various developmental stages, i.e. tadpoles and froglets.

Submitted by: Signature: Randall L. Goleman Date: 9/25/01

Authorized by: Study Director: Randall L. Goleman Date: 9/25/01

Received by: Quality Assurance Unit: [signature] Date: 3/19/02

* Sequentially numbered in order of the date that the change is effective

A FINAL REPORT

ENTITLED

PROTOCOL DEVELOPMENT FOR DETERMINATION OF EFFECTS OF PERCHLORATE ON GROWTH AND REPRODUCTION OF MOSQUITOFISH

STUDY NUMBER:	FISH 02-02
SPONSOR:	Strategic Environmental Research and Development Program (SERDP) 1155 Herndon Parkway, Suite 900 Herndon, Virginia 20170
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, Texas 79409-1163
TEST SITE:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, Texas 79409-1163
ANALYTICAL TEST SITE:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
RESEARCH INITIATION:	03/01/2001
RESEARCH COMPLETION:	12/31/2001

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GOOD LABORATORIES PRACTICES STATEMENT

Project FISH 02-02, entitled "Protocol Development for Determination of Effects of Perchlorate on Growth and Reproduction of Mosquitofish", was performed whenever possible in the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989

Submitted By:

Christopher Theodorakis, Ph.D

3/28/02
Date

1. **DESCRIPTIVE STUDY TITLE:**
Protocol Development for Determination of Effects of Perchlorate on Growth and Reproduction of Mosquitofish

2. **STUDY NUMBER:** FISH 02-02

3. **SPONSOR:**
Strategic Environmental Research
and Development Program (SERDP)
1155 Herndon Parkway, Suite 900
Herndon, Virginia 20170

4. **TESTING FACILITY NAME AND ADDRESS:**
The Institute of Environmental and Human Health
Texas Tech University
Box 41163
Lubbock, Texas 79409-1163

5. **PROPOSED EXPERIMENTAL START & TERMINATION DATES:**
Start: 03/01/2001
Termination: 12/31/2001

6. **KEY PERSONNEL:**
Christopher Theodorakis, Study Director
Todd Anderson, Analytical Chemist
Ryan Bounds, Quality Assurance Officer
Ron Kendall, Principal Investigator
Carrie Bradford, Technician
Jacques Rinchard, Technician

7. **STUDY SUMMARY:**
Different methods were tried to breed mosquitofish in the laboratory. It was found that the best way to induce breeding in the lab was by putting 4 females and 2 males together in an aquarium under conditions of 18 h light, 6 hours dark with water temperature at 25° C, 180 mg/L salinity, and 50% tank coverage with artificial plants.

Different methods were also tried to expose mosquitofish fry to perchlorate. It was found that the best way was to obtain gravid females from the wild and allow them to spawn, and then these fry were exposed to perchlorate in beakers. Fry were exposed to 0, 10, and 100 ppm for 4 weeks. Preliminary results indicate that the growth rate in the control group was slightly higher than in the test groups.

8. **STUDY OBJECTIVES / PURPOSE:**
The objective of this study was to develop protocols to determine effects of sodium perchlorate on reproduction of mosquitofish (*Gambusia holbrooki*) and the growth of fry.

9. TEST MATERIALS:

Test Chemical: Sodium Perchlorate
CAS Number : 7601-89-0
Characterization: Determination of concentration in environmental samples.
Source: EM Science

Reference Chemical: Ultrapure water with added sea salts ("Instant Ocean®")
CAS Number : NA
Characterization: Determination of pH and conductivity.
Source: City tap water that has been run through reverse osmosis and a de-ionizer to convert it to ultrapure water. 60 mg/L sea salts was added for fry exposures, 180 mg/L was added for breeding.

10. JUSTIFICATION OF TEST SYSTEM:

Ionic perchlorate alters thyroid homeostasis in fishes and amphibians as well as other vertebrates (Miranda et al., 1996; Manzon and Youson, 1997). Because of the important role played by hormones in animal development and reproduction, endocrine disruption is likely to lead to serious impairments in growth, reproductive fitness, and consequently, fish and wildlife population stability as well as human health.

Thyroid hormone has been suggested to be involved in gametogenesis and growth, and to affect fecundity in fish. Relative reproductive output and reproductive organ health are important endpoints in ecological risk assessments. Studies in the scientific literature indicate that thyroid homeostasis is important in reproductive functions such as gonadal development, growth and embryonic development. Previous studies at Texas Tech have suggested there may be effects of perchlorate on reproduction and development in zebrafish (*Danio rerio*). However, because zebrafish are not native, additional studies are needed using a species that is native to LHAAP. In order to determine effects of fecundity on these fish, it is necessary to induce the fish to breed in a highly artificial laboratory setting. Also, induction of breeding is necessary in order to expose fish during periods when reproductive effects are most likely to be manifested.

11. TEST ANIMALS:

Species: *Gambusia holbrooki*, mosquitofish
Strain: Wild
Age: Adults and fry less than 1 week old
Number: Approximately 50 adult mosquitofish and 135 fry
Source: Purchased from hatcheries
Sex: Male and Female

12. PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

The test system consisted of laboratory exposures constructed according to the experimental design described below. Identity of all fish was confirmed in the laboratory by visual inspection before tests were begun. Aquaria were labeled with the aquaria

number, species name, animal use protocol number, project number, test system, date of exposure and date of collection, concentration, and person responsible.

13. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Mosquitofish fry were exposed to two concentrations plus zero concentration or control of sodium perchlorate. Fry were placed into precleaned beakers. Beakers were cleaned by washing according to SOP AQ-1-02 "Cleaning Glassware and Aquaria for Perchlorate Assays". Beakers were located on one shelf capable of supporting such weight and the shelf held nine 1 L beakers. The experimental design consisted of randomly assigning fry to each beaker, and the beakers randomly placed on the shelf. The arrangement of the beakers was randomized in order to avoid effects due to gradients in light, temperature, volatile chemicals in the laboratory, etc. Determination of the arrangement of the beakers was done by rolling dice.

Breeding trials for adult mosquitofish were conducted in clean water only. Adult mosquitofish were placed into precleaned aquaria. Aquaria were cleaned by washing according to SOP AQ-1-02 "Cleaning Glassware and Aquaria for Perchlorate Assays". Aquaria were located either on a shelf or in an incubator. Since perchlorate was not used in these trials, aquaria were set-up right next to each other.

14. METHODS

14.1 Test System acquisition, quarantine, acclimation

Fish were obtained from fish hatcheries. Upon arrival to the lab, they were treated with commercially available antibiotics for 5 days, as instructed by the manufacturer. After five days, any debris at the bottom of the beaker or aquaria and 35% of the water was removed using a siphon hose and replaced with fresh water. Fresh water consisted of reverse osmosis (RO) water supplemented with 60 mg/L Instant Ocean sea salts for fry exposure or 180 mg/L for breeding. Fresh water was replaced in each aquaria or beaker by siphon from a reservoir (e.g., 70 gallon aquarium). Every day debris at the bottom of the beaker was cleaned by suction for the fry exposure. For breeding, debris was removed three times per week. Water was continuously aerated and filtered using mechanical and biological filtration. Animal husbandry was conducted according to SOP AQ-1-08, "General Fish Husbandry" and SOP AQ-1-09 "Mosquitofish *Gambusia spp.* Husbandry". Total acclimatization period was a minimum of one week. Once acclimated, fry were exposed to sodium perchlorate dissolved in ultrapure water. For growth of fry, gravid females were allowed to spawn and fry were collected after one week. Mosquitofish were fed commercial flake goldfish food at the rate of 5 mg per gram of fish, on a daily basis.

14.2 Test Condition Establishment

Exposures were begun after fish had become acclimatized.

14.3 Test Material Application

Mosquitofish fry were weighed and then placed into beakers containing various concentrations of sodium perchlorate, with 10 fry per beaker. Stock solutions of 10 g/L and 100 g/L sodium perchlorate in ultrapure water were used to dose the fish. The mosquitofish beakers were filled with 1 L reconstituted fresh water and an appropriate amount of stock solution was added according to the desired concentration of the aquarium water. Every day, debris was cleaned out of the aquaria and 35% of the water was replaced in each tank with reconstituted fresh water, and perchlorate stock solution was added to maintain the desired concentration.

Several different approaches have been employed in an order to assess the best strategy for inducing mosquitofish to spawn in the laboratory. First 10 males and 20 females were placed in 38 L aquaria under conditions of 18 h light, 6 hours dark with water temperature at 20° C. Because mosquitofish are livebearers that can store sperm, females can inseminate themselves for at least 3 consecutive broods. Therefore, in order to ensure that fish were not inseminated prior to beginning of experiments, two different approaches were taken. 1) Virgin fish were raised from birth with males and females being separated when secondary sexual characteristics first became evident. 2) Females were collected during the middle of winter when they are sexually inactive. Females held at temperature and photoperiod previously shown to induce spawning did not become gravid, indicating lack of stored sperm.

A second strategy involved placing 3 female and 2 male mosquitofish into 9.5 L aquaria. One group was held in a laboratory at the above photoperiod and temperature. A second group was held in a Percival incubator at the same photoperiod and at 25 or 30 ° C.

The final strategy attempted to induce breeding in the lab was by putting 4 females and 2 males together in an aquarium under conditions of 18 h light, 6 hours dark with water temperature at 25° C, 180 mg/L salinity, and 50% tank coverage with artificial plants.

Since the breeding study was only done for protocol development, these fish were not exposed to sodium perchlorate.

Rates/concentrations: Mosquitofish fry were exposed to 0, 10, and 100 ppm sodium perchlorate in water. Mosquitofish adults were not exposed to perchlorate.

Frequency: Three replicate tanks of each concentration were continually exposed four weeks. The fry exposure began on July 29, 2001 and ended

on August 27, 2001.

Route/Method of Application: Route was via dermal, oral, and respiratory exposure as the chemical was in the aquaria water.

Stock solutions for the study were mixed in precleaned glass containers as indicated in SOP AQ-1-02. Stock solutions were made by dissolving sodium perchlorate in ultrapure water, and the pH was adjusted to 7.4 (with 0.1N HCl or 0.1N NaOH, as appropriate). The appropriate amount of sodium perchlorate was weighed on a calibrated balance, and mixed into ultrapure water. The pH was checked on a calibrated pH meter (calibrated according to SOP IN-4-06) and adjusted as above. After 35% of the aquarium water had been removed and replaced (see above instructions), an appropriate amount of stock solution was added to adjust the concentration to the original value.

For the mosquitofish fry, the 10 g/L stock solution was used for the 10 ppm (10 mg/L) treatment and the 100 g/L stock was used for the 100 ppm (100 mg/L) treatment. For example, if there was 1L of water in a mosquitofish tank, 350 mL was removed and replaced every day. For the aquaria that had the 10 ppm and 100 ppm sodium perchlorate, 1 ml of 10 g/L sodoium perchlorate stock solution was added to the 10 ppm aquarium and 1 ml of 100 g/L sodium perchlorate stock solution was added to the 100 ppm aquarium initially, and 350 µL of 10 g/L and 350 µL of 100 g/L perchlorate stock solution, respectively, was added following water changes (e.g., (1L x 0.01 g/L)/ 10 g/L = 0.001 L (1 mL), 35% of this was replaced after each water change). Stock solutions were measured out in an appropriate pipette.

Justification for Exposure Route: Exposure by environmental waters is most appropriate because fish respire, ingest, and are dermally exposed to chemicals in the waters in which they live.

Exposure Verification: A sample of each concentration of treated water was collected at the beginning of the exposure period and when fish were removed from the aquarium for analysis. A sample was also collected once a week during the fry exposure. The concentration of perchlorate in the water was tested using ion chromatography.

14.4 Test System Observation

Aquaria were observed on a daily basis. The number of individuals that expired each day as well as any abnormal behavior was recorded for each perchlorate concentration throughout the fry exposure. Evidence of reproductive behavior and pregnancy was observed in adult mosquitofish. In addition, pH, dissolved oxygen, conductivity, ammonia, and

temperature were determined at least 3 times per week.

14.5 Animal Sacrifice and Sample Collections

Mosquitofish fry were removed from treated aquaria and then rinsed three times in aquaria with reconstituted fresh water. The fish were sacrificed with 1 g/L MS 222 until gill ventilation ceased and the fish did not respond to physical stimuli, according to SOP AQ-1-03 "MS-222 Anesthesia and Euthanasia of Amphibians and Fish". The weight of each fish was recorded. The fish were then preserved in Bouin's fixative (a mixture of 1.5 L picric acid, 0.5L 37% formalin and 0.1L glacial acetic acid) in scintillation vials for future analysis.

For the adults, no samples were collected but mating behavior and pregnancy were observed.

14.6 Endpoint Analysis

The growth rate was used as an endpoint for the fry exposures. The growth rate was determined by [(final weight-initial weight)/initial weight] x 100 to obtain the % mass gain.

Mating behavior and pregnancy were used as the endpoint for the adult breeding studies.

15. STATISTICAL METHODS

Since this was a pilot study for protocol development, no statistical tests were conducted.

16. PROTOCOL CHANGES/REVISIONS:

Since this study was conducted for protocol development, no changes were necessary.

17. RESULTS

For the mosquitofish housed in 38 L aquaria, none of the virgin females showed signs of being gravid after 4 weeks, while many of the adult fish collected during non-breeding season did show signs of being gravid after 4 weeks. Gravidity was determined by distention of the female's abdomen and enlargement of the "brood spot", a black spot at the posterior abdomen ventral to the midline, which enlarges when females are gravid. For the fish kept in 9.5 L aquaria, none of the females appeared to be gravid, and many of the fish kept in the incubator died. The only successful method found to induce breeding in the lab was by putting 4 females and 2 males together in an aquarium under conditions of 18 h light, 6 hours dark with water temperature at 25° C, 180 mg/L salinity, and 50% tank coverage with artificial plants.

The growth rate, as indicated by percent increase, was slightly greater in the 0 ppm juveniles than in the 10 or 100 ppm juveniles (Table 1). The differences were not statistically significant however, but this may have been due to the low statistical power

afforded by the extremely small sample size of this pilot study (n=3).

Table 1. Mean (± SD) mass (g) of juvenile mosquitofish exposed to sodium perchlorate for 4 weeks.

[a][(Initial mass – final mass)/initial mass] x 100

	Treatment		
	0 ppm	10 ppm	100 ppm
Initial mass	0.080 ± 0.015	0.074 ± 0.001	0.070 ± 0.013
Final mass	0.101 ± 0.030	0.080 ± 0.015	0.083 ± 0.010
% Difference[a]	27.7 ± 22.0	20.3 ± 12.7	21.6 ± 14.0

The project milestones and deliverables were stated in the original proposal were development of an exposure protocol and generation of preliminary data. This has been accomplished.

18. DISCUSSION

It was found that the best way to induce breeding in the lab was by putting 4 females and 2 males together in an aquarium under conditions of 18 h light, 6 hours dark with water temperature at 25° C, 180 mg/L salinity, and 50% tank coverage with artificial plants. In previous breeding trials without the presence of artificial plants there was no evidence of breeding, even after four weeks.

For looking at the effects of perchlorate on fry survival and growth, it was decided that the best experimental design was to obtain gravid females from the wild and allow them to spawn in the lab, and then after one week expose these fry to perchlorate. The method of exposure involved randomly assigning 10 fry to 1 L beakers and exposing them to sodium perchlorate for 4 weeks. Initial and final weights of the fry as well as daily mortality records are maintained for analysis.

Based upon these recommendations, a protocol was developed for assessing the effects of perchlorate on growth, reproduction, and survival of mosquitofish (Appendix 1).

19. STUDY RECORDS AND ARCHIVE:

Study Records will be maintained at The Institute of Environmental and Human Health (TIEHH) Archive for a minimum of one year after study completion date.

20. REFERENCES:

Manzon RG and Youson JH. 1997. Immunocytochemical and morphometric study of TSH, PRL, GH, and ACTH cells in Bufo arenarum larvae with inhibited thyroid function. Gen. Comp. Endocrinol. 98: 166-176.

Miranda, LA, Paz, DA, Dezi, RE and Pisano, A. 1996. Immunocytochemical and morphometric study of TSH, PRL, GH, and ACTH cells in Bufo arenarum larvae with inhibited thyroid function. Gen. Comp. Endocrinol. 98: 166-176.

21. APPENDICES:

Study Protocol

Project No. T9700.11

A STUDY PROTOCOL

ENTITLED

Effects of Perchlorate on Growth, Reproduction, and Survival of Mosquitofish

STUDY NUMBER: FISH-02-02

SPONSOR: United States Air Force
IERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

ADMINISTRATOR: The Institute of Environmental and Human Health

TESTING FACILITY

Name/Address:

The Institute of Environmental and Human Health
Texas Tech University/Texas Tech University Health Sciences Center
PO Box 41163
Lubbock, Texas 79409-1163

Test Facility Management: Dr. Ronald Kendall

Study Director: Dr. Christopher Theodorakis

PROPOSED EXPERIMENTAL
START DATE: MARCH 1, 2002

1. **DESCRIPTIVE STUDY TITLE:** Effects of Perchlorate on Growth, Reproduction, and Survival in Mosquitofish

2. **STUDY NUMBER:** FISH-02-02

3. **SPONSOR:** United States Air Force
IERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

4. **TESTING FACILITY NAME & ADDRESS:**
The Institute of Environmental and Human Health
Texas Tech University
PO Box 41163
Lubbock, Texas 79409-1163

5. **PROPOSED EXPERIMENTAL START & TERMINATION DATES:**
Start Date: (date of chemical application) March 1, 2002

Termination Date: (date of last data collected) September 31, 2002

6. **KEY PERSONNEL:**
Christopher Theodorakis, Study Director
Ronald Kendall, Testing Facility Management
Todd Anderson, Analytical Chemist
Ryan Bounds, Quality Assurance Manager

7. **DATED SIGNATURES:**

_____ _____ Dr. Christopher Theodorakis
Study Director

_____ _____ Dr. Ronald Kendall
Testing Facility Management

_____ _____ Mr. Ryan Bounds
Quality Assurance Manager

_____ _____ Dr. Todd Anderson
Analytical Chemist

8. REGULATORY COMPLIANCE STATEMENT:

Quality Control and Quality Assurance

This study will be conducted in accordance with established Quality Assurance program guidelines and in compliance, where appropriate and possible, with Good Laboratory Practice Standards (40 CFR Part 160, August 17, 1989).

Document Control Statement

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Dr. Ronald Kendall
The Institute of Environmental and Human Health
Texas Tech University
PO Box 41163
Lubbock, Texas 79409-1163

9. STUDY OBJECTIVES / PURPOSE:

To determine the effects of perchlorate on reproduction of adult mosquitofish and growth and survival of mosquitofish fry.

10. TEST MATERIALS:

Test Chemical name: Sodium perchlorate
CAS number: 77601-89-0
Characterization: Determination of concentration in environmental samples.
Source: EM Science

Reference Chemical name: ultrapure water with added sea salts ("Instant Ocean®" or any other brand of sea salts with identical or nearly identical composition).
CAS Number: Not applicable
Characterization: Determination of pH and conductivity
Source: City tap water that has been run through reverse osmosis and a de-ionizer to convert it to ultrapure water. 60 mg/L sea salts will be added for fry exposures and 180 mg/L sea salts will be added for breeding exposures.

11. JUSTIFICATION OF TEST SYSTEM:

Ionic perchlorate alters thyroid homeostasis in fishes and amphibians as well as other vertebrates (Miranda et al., 1996; Manzon and Youson, 1997). Because of the important role played by hormones in animal development and reproduction, endocrine disruption is

likely to lead to serious impairments in growth, reproductive fitness, and consequently, fish and wildlife population stability as well as human health.

12. **TEST ANIMALS** (Where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test system)**:**

Species: *Gambusia holbrooki*, mosquitofish

Strain: Feral organisms or bred in hatcheries

Age: Adults and fry less than 1 week old.

Number: Approximately 120 adult mosquitofish (80 female and 40 male) and 750 fry

Source: Captured in the wild, or purchased from hatcheries, Carolina Biological Supply or other commercial suppliers

13. **PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:**
The test system will consist of laboratory exposures constructed according to the experimental design described below. Wild fish will be identified in the field (upon capture) by the project manager or personel trained in the identification of such fish. Identity of all fish will be confirmed in the laboratory by visual inspection before tests are begun. Aquaria will be labeled with the aquaria number, species name, animal use protocol number, project number, test system, date of exposure and date of collection, concentration, and person responsible.

14. **EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:**
Mosquitofish fry will be exposed to five concentrations of sodium perchlorate plus zero control for survival study, three concentrations of sodium perchlorate plus zero control for growth study, and mosquitofish adults will be exposed to three concentrations of sodium perchlorate plus zero control for reproductive study. Fish will be placed into precleaned aquaria or beakers. Aquaria and beakers will be cleaned by washing according to SOP AQ-1-02 "Cleaning Glassware and Aquaria for Perchlorate Assays". For exposures, aquaria or beakers will be located on shelves capable of supporting such weight. Each shelf will hold all the 1 L beakers (for the fry survival and growth studies) or four 20L aquaria (for the adult reproductive study). The experimental design will consist of a randomized block design, with each shelf constituting a block for the adult exposures. The arrangement of the aquaria within each block will be randomized in order to avoid effects due to gradients in light, temperature, volatile chemicals in the laboratory, etc. Determination of the arrangement of the aquaria or beakers within each block by a

random number generator, random number table or by rolling dice. Each block will contain at least 1 aquarium or beaker of each treatment. Fish will be placed in the aquaria in random order, within blocks, using the procedure described below.

For the adult mosquitofish, each block will consist of 4 aquaria, each with 4 female and 2 male fish. There will be 5 blocks, for a total of 120 fish. All female fish will be placed in one or more aquaria and all male fish will be paced in one or more aquaria. Within each block, each aquarium will be assigned a number from 1-4. A random number generator will be used to randomly order the numbers 1-4, and this will be done 4 times for the female fish and 2 times for the male fish: e.g., 3412, 4123, 4312, 4213 (female fish) 3421, 1342 (male fish). A pair of dice, a random number table, or a computerized random number generator can be used for this purpose. The fish will be placed into the 4 aquaria in the 1st block according to this list of numbers. For example, one female fish will be placed into aquarium 3, then aquarium 4, aquarium 1, and finally aquarium 2. A second female fish will be placed in each aquarium in the order 4123. A third female fish will then be placed in each aquarium in the order 4312. Finally a fourth female fish will be paced in each aquarium in the order 4213. Male fish will be placed in each aquarium in the order 3412, then 1342. This will then be done for the other 4 blocks in the experiment.

For the fry mosquitofish, all beakers will be on one shelf in random order. Ten fry will be randomly assigned to each beaker in a similar manner as described above.

15. METHODS:

15.1 Test System acquisition, quarantine, acclimation

Fish will be obtained from the wild populations, commercial vendors, or fish hatcheries. If fish are captured in the wild, they will be transported back from the field in plastic buckets or other containers with constant aeration. Upon arrival to the lab, they will be treated commercially available antibiotics for 5 days, as instructed by the manufacturer. After five days, any debris at the bottom of the tank and 1/3 of the tank water will be removed using a siphon hose or electric pump and replaced with fresh water. Fresh water will consist of reverse osmosis (RO) water supplemented with 60 mg/L (for fry exposures) or 180 mg/L (for adult exposures) Instant Ocean sea salts or other brands of identical composition. Fresh water will be replaced in each tank by siphon or electric pump from a reservoir (e.g., 100 gallon aquarium). Each day, debris at the bottom of the tank will be cleaned by suction. Water will be continuously aerated and filtered using mechanical and biological filtration. Animal husbandry will be according to SOP AQ-1-08, "General Fish Husbandry" and SOP AQ-1-09 "Mosquitofish *Gambusia spp.* Husbandry". Total acclimatization period will be a minimum of one week. Once acclimated, fish will be exposed to sodium perchlorate dissolved in water. Mosquitofish will be fed commercial flake goldfish food at the rate of 5 mg per gram of fish, on a daily

basis. Debris and uneaten food will be removed from the bottom of the tank 1-3 hours post feeding.

Gravid females will be obtained from hatcheries and fry will be collected according to SOP AQ-1-09 "Mosquitofish *Gambusia spp.* Husbandry". All females will be placed in one 80 L aquaria and fry will be collected for one week. Fry that are less than 1 week old will be used for fry survival and growth experiments.

15.2 **Test Condition Establishment**

Exposures will begin after fish have become acclimatized.

15.3 **Test Material Application**

Fish will be placed into beakers or aquaria containing various concentrations of sodium perchlorate, with 10 fish per beaker and 5 replicate beaker per treatment (for fry exposures) or 6 fish per aquaria and 5 replicate aquaria per treatment (for adult exposure). Stock solutions of 1 g/L, 10 g/L, 100 g/L and 1,000 g/L perchlorate in reconstituted fresh water will be used to dose the fish. The mosquitofish aquaria will be filled with 15 L of reconstituted fresh water, and the mosquitofish beakers will be filled with 1 L of reconstituted fresh water, and an appropriate amount of stock solution will be added according to the desired concentration of the aquarium or beaker water. Every other day, debris will be cleaned out of the aquaria and 1/3 of the water will be replaced in each tank with undosed water (as described in 15.1), and perchlorate stock solution will be added to maintain the desired concentration.

For fry survival, ten fry will be randomly assigned to each 1 L beaker and immediately exposed to 0, 100, 500, 1000, 2000, and 3000 ppm sodium perchlorate for five days. Fish will be feed according to SOP AQ-1-09 "Mosquitofish *Gambusia spp.* Husbandry."

For fry growth, ten fry will be randomly assigned to each 1 L beaker and will be weighed prior to placement in beakers. Fry will be continuously exposed to 0, 1, 10, and 100 ppm sodium perchlorate in water for 4 weeks. Fish will be feed according to SOP AQ-1-09 "Mosquitofish *Gambusia spp.* Husbandry."

For adult reproduction, four females and two males will be randomly assigned to each aquaria and continuously exposed to 0, 1, 10, and 100 pppm sodium perchlorate in water for six weeks in aquaria conditions of 18 h light, 6 h dark with water temperature at 25°C, 180 mg/L salinity, and 50% tank coverage with artificial plants. A 0.5 cm thick layer of activated charcoal will be added to the filtration unit to remove any contaminants released from the artificial plants.

Rates/concentrations: Mosquitofish will be exposed to 0, 100,500, 1000, 2000, and

3000 ppm (for fry survival) or 0, 1, 10, and 100 ppm (for fry growth) or 0, 1, 10, and 100 ppm (for adult reproduction) sodium perchlorate in water.

Frequency: Five replicate tanks of each concentration will be continually exposed for 5 days for determination of fry survival, 4 weeks for determination of fry growth, and 6 weeks for adult reproduction. Exposure periods may be extended if initial results warrant.

Route/Method of Application: Route will be via dermal, oral and respiratory exposure as the chemical will be in the beaker/aquaria water.

Stock solutions for study will be mixed in precleaned glass containers as indicated in SOP AQ-1-02-01. Stock solutions will be made by dissolving sodium perchlorate in reconstituted fresh water (60 mg/L Instant Ocean® sea salts or equivalent, in ultrapure water, pH adjusted to 7.4 with 1N HCl or 1N NaOH, as appropriate). The appropriate amount of sodium perchlorate compound will be weighed on a calibrated balance, and mixed into reconstituted fresh water. The pH will be checked on a calibrated pH meter (calibrate according to SOP IN-4-06) and adjusted, if necessary, as above. After 1/3 of the aquarium water has been removed and replaced (see above instructions), an appropriate amount of stock solution will be added to adjust the concentration to the original value.

For the adult mosquitofish, the 10 g/L stock will be used for the 1 ppm (1 mg/L) and 10 ppm (10 mg/L), treatments and the 100 g/L stock will be used for the 100 (100 mg/L) treatments. For example, if there is 15L water in a mosquitofish tank, 5L will be removed and replaced every other day. For the aquaria that have the 1 ppm and 10 ppm, 0.0015 L (1.5 ml) of perchlorate stock solution will be added to the 1 ppm aquarium and 0.015 L (15 ml) will be added to the 10 ppm aquarium initially , and 0.5 mL and 5 mL of perchlorate stock solution will be added following water changes (e.g., (15L x 10 mg/L)/10,000 mg/L = 0.015 L, one-third of this will be replaced after each water change). Stock solutions will be measured out in an appropriate pipette.

For the fry exposures, the 1 g/L stock will be used for the 1 ppm (1mg/L), the 10 g/L stock will be used for the 10 ppm (10 mg/L), the 100 g/L will be used for the 100 ppm (100 mg/L), and the 1,000 g/L stock will be used for the 1000 ppm (1000 mg/L), 2000 ppm (2000 mg/L), and 3000 ppm (3000 mg/L) treatments.

Justification for Exposure Route: Exposure by environmental waters is most appropriate because fish respire, ingest, and are dermally exposed to chemicals in the waters in which they live.

Exposure Verification: A sample of each concentration of treated water will be collected on the first day of exposure and whenever animals are removed from the aquarium for analysis. Water samples will also be collected periodically throughout the exposure period (at least once per week). The concentration of perchlorate in the water will be tested using ion chromatography.

15.4 **Test System Observation**

Tanks or beakers will be observed on a daily basis. The number of individuals that expire each day will be recorded for each perchlorate concentration as well as any abnormal behavior. In addition, pH, dissolved oxygen, conductivity, temperature, ammonia, and any other water chemistry parameters deemed appropriate by the project manager will be determined at least 3 times per week.

15.5 **Animal Sacrifice and Sample Collections**

Fry used to determine survival will be euthanized with 1.0 g/L MS-222 and the number of surviving fry following exposure to sodium perchlorate will be recorded. Fry used to determine growth rate will be euthanized in 1.0 g/L MS-222 and the final weight will be recorded. Adult mosquitofish will be euthanized in 1.0 g/L MS-222 and the females will be dissected. The ovaries will be removed and the eggs will be put in water. The egg volume will be determined by measuring the change in water volume divided by the number of eggs. All mosquitofish will then either be disposed or preserved for future analysis.

15.6 **Endpoint Analysis**

For fry survival, the number of survivors will be counted as the endpoint. For fry growth, percent mass gain will be used as the endpoint [(final weight-initial weight)/initial weight] x 100. For adult reproduction, fecundity as expressed by the ratio of number of eggs to body weight will be used as the endpoint.

16. **PROPOSED STATISTICAL METHODS:**

ANOVA or nonparametric analysis such as Kruskal-Wallis will be used to determine effects of perchlorate on fry growth and reproduction. Probit analysis to test for LC_{50} will be used to determine effects of perchlorate on fry survival.

17. **REPORT CONTENT/RECORDS TO BE MAINTAINED:**

Records to be maintained include: Room temperature and water temperature, dissolved oxygen, salinity, ammonia, and pH will be collected. Date, time, and amount of feedings per tank will be recorded. Perchlorate effects on fry survival and growth and on adult reproduction will be included in the final report.

Report content will include presentation of data, interpretation, and discussion of the

following endpoints:

List individual endpoints and analyses.
Interpretation of all data, including statistical results
Discussion of the relevance of findings
List of all SOPs used
List of all personnel

18. **RECORDS TO BE MAINTAINED / LOCATION:**

The final report will be delivered to the Sponsor on or before December 31, 2002. Copies of all data, documentation, records, protocol information, as well as the specimens shall be sent to the Sponsor, or designated delivery point, upon request. All data, the protocol and a copy of the final report shall be archived at the testing facility.

19. **QUALITY ASSURANCE:**

The Quality Assurance Unit will inspect the study at intervals to insure the integrity of the study. Written records will be maintained indicating but not limited to the following: date of inspection, study inspected, phase inspected, person conducting the inspection, findings and problems, recommended and taken action, and any scheduled re-inspections. Any problems likely to effect study integrity shall be brought to the immediate attention of the Study Director. The Quality Assurance Unit will periodically submit written status reports on the study to management and the Study Director.

20. **PROTOCOL CHANGES / REVISIONS:**

All changes and/or revisions to the protocol, and the reasons therefore, shall be documented, signed and dated by the Study Director and Test Facility Manager and maintained with the protocol and the Quality Assurance Unit.

21. **REFERENCES:**

Manzon RG and Youson JH. 1997. Immunocytochemical and morphometric study of TSH, PRL, GH, and ACTH cells in Bufo arenarum larvae with inhibited thyroid function. Gen. Comp. Endocrinol. 98: 166-176.

Miranda, LA, Paz, DA, Dezi, RE and Pisano, A. 1996. Immunocytochemical and morphometric study of TSH, PRL, GH, and ACTH cells in Bufo arenarum larvae with inhibited thyroid function. Gen. Comp. Endocrinol. 98: 166-176.

A FINAL REPORT

ENTITLED

Assessment of Perchlorate in Terrestrial Mammalian Receptors: Raccoons (*Procyon lotor*) and Opossums (*Didelphis virginiana*)

STUDY NUMBER:	CAD-00-01
SPONSOR:	Strategic Environmental Research and Development Program (SERDP) 1155 Herndon Parkway, Suite 900 Herndon, Virginia 20170
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University/TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, Texas 79409-1163
TEST SITE:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, Texas 79409-1163
ANALYTICAL TEST SITE:	The Institute of Environmental and Human Health Texas Tech University/TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
RESEARCH INITIATION:	April 2000
RESEARCH COMPLETION:	December 2001

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List of Tables and Figures

GOOD LABOARATORIES PRACTICES STATEMENT

Project T97001.6 titled "Assessment of Perchlorate in Terrestrial Mammalian Receptors: Raccoons (*Procyon lotor*) and Opossums (*Didelphis virginiana*) ", was performed whenever possible in the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Noted exceptions are as follows:

Submitted By:

Philip N. Smith, Ph.D.

3/28/02
Date

QUALITY ASSURANCE STATEMENT

This study was conducted under the Institute of Environmental and Human Health's Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Any changes in protocol and SOPs were documented in writing and signed by the study director.

The Quality Assurance Officer verbally notified the Study Director of all findings at the time of the inspection. Written audit reports were also submitted to the Study Director and Test Facility Management. Audits were performed for the following phases of the project:

Auditable Research Phase / Activity	Audit Dates		Date written report submitted to Study Director	Date written report submitted to Management
	Start	End		
Total T4 RIA on Raccoon Samples	08/10/01	08/10/01	08/13/01	08/13/01
TSH RIA on Raccoon Samples	09/27/01	09/28/01	10/01/01	10/01/01
Final Report and Raw Data Review	03/08/02	03/22/02	03/28/02	

Submitted By:

Ryan Bounds
Quality Assurance Manager

03/28/02
Date

1.0 DESCRIPTIVE STUDY TITLE:

Assessment of Perchlorate in Terrestrial Mammalian Receptors: Raccoons (*Procyon lotor*) and Opossums (*Didelphis virginiana*)

2.0 STUDY/PROTOCOL NUMBER: CAD-00-01

3.0 SPONSOR:

Strategic Environmental Research
and Development Program (SERDP)
1155 Herndon Parkway, Suite 900
Herndon, VA 20170

4.0 CONTRACT ADMINISTRATOR:

The Institute of Environmental and Human Health
Texas Tech University / TTU Health Science Center
Box 41163
Lubbock, TX 79409-1163

5.0 TESTING FACILITY NAME & ADDRESS:

The Institute of Environmental & Human Health
Texas Tech University / Texas Tech University Health Sciences Center
Box 41163
Lubbock, TX 79409-1163

6.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:

Start Date: April 1, 2000
Termination Date: December 31, 2001

7.0 KEY PERSONNEL:

Dr. Philip N. Smith, Study Director
Dr. Scott T. McMurry, Study Advisor

Ms. Sarah J. Utley, Research Assistant
Mr. Ryan M. Bounds, Quality Assurance Officer
Dr. Ronald J. Kendall, Primary Investigator / Testing Facility Management

8.0 STUDY OBJECTIVES /TEST SYSTEM JUSTIFICATION:

Analytical evaluation of ground and surface water, soil, and sediment samples taken from within the LHAAP boundaries have indicated that perchlorate contamination at the Longhorn Army Ammunition Plant (LHAAP) is bioavailable to terrestrial organisms (Smith et al., 2001). Detectable perchlorate concentrations have ranged from 4 to 31,438 ppb in water and 44 to 35,630 ppb in sediment samples (Smith et al., 2001).

The presence of perchlorate at LHAAP is the result of past military activities. Several locations within the LHAAP have been associated with perchlorate handling, maintenance, or detonation (2 burning grounds, a water treatment holding pond, and Building 25C). Caddo Lake, Goose Prairie Creek, Central Creek, and the Harrison Bayou drainage system are at risk of perchlorate contamination through erosion, run-off and groundwater movement.

Raccoons (*Procyon loctor*) are a terrestrial mammal closely associated with the terrestrial/aquatic interface and are common throughout the LHAAP. Raccoons consume approximately 0.083 g water/day and their diet is principally composed of plant and animal matter. Perchlorate water concentrations at the LHAAP are variable, as detected concentrations have been regularly detected in the range of 4 to 776 ppb. Based on these perchlorate concentrations raccoons can potentially ingest 124-289 ug/day from water alone.

Exposure could also occur from consumption of contaminated food items. LHAAP raccoons likely consume insects, amphibians, crayfish, fish, berries, rodents and various vegetation including soft mast present on site. Perchlorate has been detected in each of these food sources at various levels. Specifically, concentrations have ranged from 1130-2567ppb in tadpoles, ND to 580 ppb in frogs, ND to 207 ppb in fish, ND to 5,557,000 in vegetation, and up to 593,497 ppb in black berries (Smith et al, 2001; TIEHH Data on file).

It is also estimated that raccoon diets are composed of 9.4% soil (from feeding and grooming) (Beyer et al, 1994). Soil perchlorate concentrations at the LHAAP site have ranged from below detectable levels to 35,630 ppb. Based on potential for exposure through water ingestion, diet, and soil consumption, raccoons were selected as a sentinel species for this study. Opossums (*Didelphis virginiana*) were also targeted for capture because they occupy similar habitats and have similar foraging strategies.

Perchlorate interferes with thyroid function (Wolff, 1998). It has been used pharmacologically to treat hyperthyroidism as it decreases the active transport of iodide into the thyroid (Wolff, 1998). Thyroid hormone formation is dependent on the transport

of iodide from extracellular fluid into thyroid glandular cells and follicles (Norris, 1997). Through a process called iodide trapping the thyroid cell transports iodide actively into the cell at a concentration of about 30 times its concentration in the blood (Norris, 1997). Once in the thyroid cell, iodine is oxidized and binds with the amino acid tyrosine (Norris, 1997). Tyrosine is first oxidized to monoiodotyrosine and then to diiodotyrosine, which couple with one another (Norris, 1997). Two diiodotyrosines couple with each other to form thyroxine (T4) (Norris, 1997). One molecule of monoiodotyrosine and one molecule of diiodotyrosine couple with one another to form triiodothyronine (T3) (Norris, 1997).

Perchlorate interferes with iodide accumulation in the thyroid by competitive inhibition of the sodium-iodide symporter (NIS), the thyroid cell surface protein which is responsible for transporting iodide from the plasma into the thyroid, thereby blocking hormone production and output. This results in reduced excretion of thyroid hormones followed by a feedback loop that results in increased secretion of thyroid stimulating hormone by the pituitary gland.

The hypotheses for this study were based on what we knew about the LHAAP site, the foraging behavior of raccoons, and the known effects of perchlorate. First, we hypothesized that we would detect perchlorate in raccoon plasma resulting from exposure to perchlorate on site. Second, we hypothesized that raccoon thyroid hormone concentrations would be altered from perchlorate exposure. Due to perchlorate's competitive inhibition with iodide accumulation we expected to see decreases in T3 and T4 hormone concentrations and increases in TSH concentrations to the feedback regulation of the thyroid gland. We hypothesized that the spatial distribution of raccoon exposure and effects would be related to the distribution of perchlorate contamination at LHAAP. Finally, we attempted to identify potential perchlorate related developmental anomalies by direct examination of opossum embryos.

9.0 TEST ANIMALS (number, weight, source, strain):

Species: Raccoons (*Procyon lotor*), Opossums (*Didelphis virginiana*)

Strain: Wild

Age: Adult

Number: Forty-four raccoons and eight opossums

Source: Collected/captured on or near LHAAP, Karnack, Texas

10.0 METHODS:

Sample Collection and Field Procedures

Raccoons and opossums were collected (live-trapped) from areas with varying perchlorate contamination levels within the LHAAP. Traps were baited with sardines, or cat food and placed at locations frequented by raccoons (as noted by tracks, scat etc.). Traps were covered with vegetation to help prevent exposure to adverse environmental conditions and to help conceal the trap. Since raccoons are mostly nocturnal, traps were set in the evening, and checked the following morning. Raccoons that were lactating were released and all others were held briefly for sample collection.

Upon capture, raccoons were transferred to pre-weighed squeeze cages and weighed using a pesola scale calibrated to the nearest 0.5 kg. The individual's weight was recorded and used to calculate the appropriate dose of ketamine hydrochloride (8-10 mg/kg) and xylazine hydrochloride (2mg/kg) for sedation purposes. The sedative was administered via injection while raccoons were constrained in squeeze cages. Sedation was deemed adequate upon the loss of the "righting reflex"; additional sedative was administered if the original injection was insufficient. Ophthalmic ointment was applied to the eyes to keep them moist.

Raccoons were sexed, ear tagged, and examined for general health and reproductive condition. All animals were tagged with one Monel 1005-4 ear tag (brass-aluminum style 893, National Tag and Brand Co.) in each ear (lower outer portion) for identification. Males (scrotal or nonscrotal) and females (pregnant, lactating, open) were examined for reproduction status. Raccoons were aged (juvenile or adult) and a general health examination noted overall body condition (good, fair, poor).

Blood samples were then drawn (approximately 3 to 8 ml, depending on body size) from the jugular vein, placed in plasma tubes, and centrifuged on an IED MediSpin centrifuge at maximum speed for 12 minutes or until the serum and plasma separated. Samples were frozen (-80°C) until hormone and residue analysis. Animals were monitored to ensure full recovery from anesthesia and released at their capture location.
Potential raccoon food items and water samples were collected over the course of the study. These included soil, water, berries, plant matter, fish, amphibians and insects. Samples were taken at locations throughout LHAAP, including those known to be contaminated.

Radio-telemetric monitoring of raccoons was conducted during the study to evaluate spatial exposure and responses (TIEHH SOP ET 3-02). Select raccoons were fitted with a radio-collar and tracked during the study to evaluate spatial utilization of contaminated areas.

Analytical Sample Analysis - Perchlorate Concentration

Raccoon plasma was analyzed to determine if perchlorate could be detected in raccoon blood. Samples were prepared for analysis by adding 5ml ethanol, centrifuging for 10 minutes, and decanting the supernatant. The supernatant was analyzed for perchlorate

with Ion Chromatography (dionex DX 500 equipped with a conductivity detector) (TIEHH SOP AC-2-11).

Sample Analysis - Hormone Concentration (T3, T4, and TSH)
Samples were analyzed for T3, T4, TSH, and perchlorate, subject to the availability of plasma from individual raccoons. If the plasma volume of an individual raccoon was insufficient for all four tests, priority order was perchlorate residue analysis, followed by, T3 analysis, T4 analysis, and then TSH analysis.

Diagnostic Products Coat - A- Count Total kits were used to measure T3 and T4 levels, and the Biotrak Rat Thyroid Stimulating Hormone [rTSH] Assay System was used to measure TSH levels. Standard curves and plasma volumes were optimized to measure T3, T4, and TSH in raccoon plasma. In addition, calibration points were added to the TSH standard curve at critical locations on the curve (the most linear section). In order to conserve plasma collected from the LHAAP raccoon population, all kit optimization was completed using raccoon serum collected during a previous study.

The Coat-A-Count Total T3 and Total T4 tests are solid-phase radioimmunoassays based on antibody coated tubes and human serum calibrators. ^{125}I labeled hormone (T3 or T4) compete with unlabeled hormones (raccoon T3 or T4) in the sample for antibody binding sites on the coated tubes. This reaction takes place in the presence of blocking agents that serve to liberate the bound hormone from carrier proteins. Therefore, total T3 or T4 was measured, since both free and protein-bound T3 (or T4) from the sample are able to compete with radiolabeled T3 (or T4) for antibody sites. The antibody-hormone complex is adhered to the wall of the polypropylene tube and can thus be isolated by simply decanting the supernatant.

The rat thyroid stimulating hormone assay (rTSH) utilizes ^{125}I as a tracer and a highly specific antiserum. Separation of the antibody bound fraction from the free fraction is achieved with a second-antibody, allowing a centrifugal separation. The assay is based on the competition between unlabelled raccoon TSH and a fixed quantity of ^{125}I-labelled rTSH for a limited number of binding sites on an rTSH specific antibody. Since there are a fixed amount of antibodies and radioactive ligands, the amount of radioactive ligands bound by the antibodies will be inversely proportional to the concentration of added non-radioactive ligands. The antibody bound raccoon TSH is then reacted with the second antibody reagent that contains a second antibody that is bound to magnetic polymer particles. Separation of the bound fraction is completed by centrifugation and decantation of the supernatant. Measurement of the radioactivity in the bound fraction is a determination of the labeled raccoon TSH. The concentration of unlabelled rTSH in the sample is then determined from the standard curve.

Radioimmunoassay Analysis - Quality Assurance
All original samples were tested simultaneously to eliminate any inter-assay variation. Reference samples and internal standards were used for additional quality assurance. Additionally, standards were run in triplicate to increase the accuracy of the curve.

Coefficient of variation (CV) and percent error were calculated for each individual. Any samples with a CV above 15% were tested again.

In addition to the individual hormone tests, a dilution curve test was performed on all three-hormone assays. In this assay, varying amounts of plasma were tested in each individual kit. Upon completion of the test, one would expect to see corresponding hormone concentrations with varying plasma concentrations.

Statistical Methods

Correlations among thyroid hormone concentrations were evaluated using Pearson's product moment. ANOVA techniques were used to evaluate differences between male and female thyroid hormone concentrations.

11.0 RESULTS

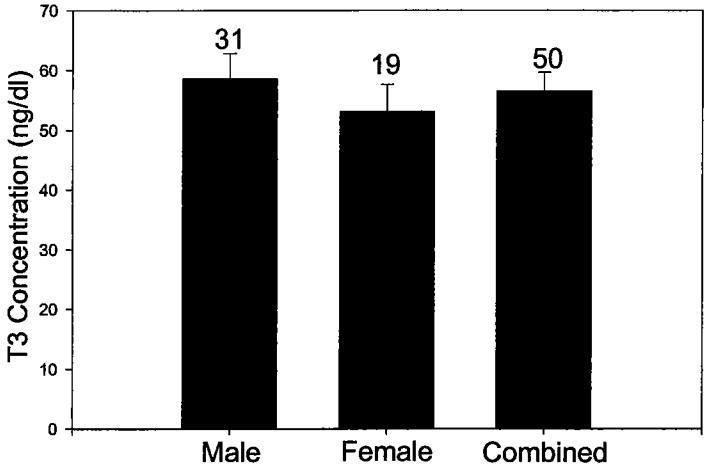
Forty-four individual raccoons were captured over the course of this study; eleven of which were recaptures (ten were captured twice and one captured three times). Therefore, there were a total of fifty-six capture events. Plasma was not collected during six capture events. Very few female opossums with embryos were captured. Therefore, we were unable to evaluate developmental anomalies as planned.

Fifty raccoon plasma samples were analyzed for hormone concentrations and perchlorate residue. For the T3, T4, and perchlorate residue analysis assays there were five sets of duplicates and one set of three samples from recaptures. There were two sets of duplicates and one set of triplicates from recaptures in the TSH assay.

T3 Hormone Concentration Analysis

The final plasma volume for determining T3 was 150µl. The T3 concentration range for the combined populations is relatively normal and only has one outlier from the general population (13.0ng/dl to 107.2ng/dl). The mean T3 hormone concentration for the total population was 56.5±3.1ng/dl. There were no significant differences (P=0.21) between the mean concentration of T3 for males and females (males 58.6±4.2ng/dl; females 53.1±4.5ng/dl). Calculated T3 hormone values are contained in Table 1. Averages from male, female, and combined concentration values are contained in Figure 1.

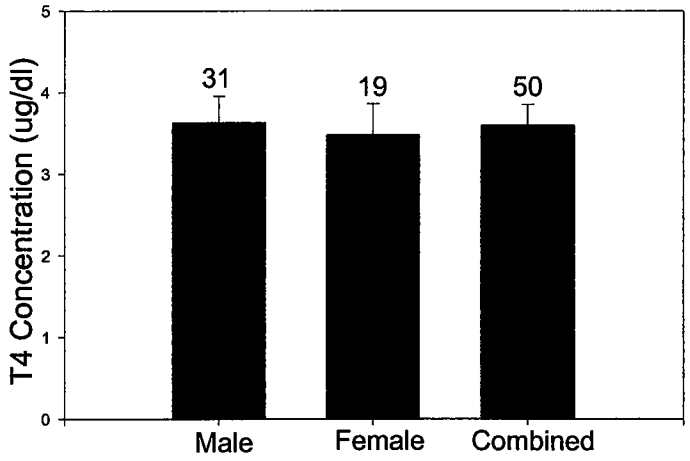
Figure 1: T3 Mean Concentration Values



T4 Hormone Concentration Analysis

The final plasma volume for determining T4 was 100µl. Differences in mean concentration of T4 between males and females were not statistically different (males 3.63±0.3µg/dl; females 3.48±0.38µg/dl (P=0.27)). The mean concentration value for the total population was 3.57±0.25µg/dl and ranged from below detectable limits to 8.6µg/dl. Calculated values relating the T4 mean, range and standard error are contained in Table 1. Averages from male, female and combined concentration values are contained in Figure 2.

Figure 2: T4 Mean Concentration Values



TSH Hormone Concentration Analysis

The final plasma volume for determining TSH was 175µl. The differences in male and female TSH concentrations were not statistically significant (P=0.72). Females had a mean concentration of 1.07±0.08ng/ml and males had a mean concentration of 0.945±0.08ng/ml. Averages from males, females and the sexes combined are contained in Figure 3 and mean, range and standard error values are in Table 1.

Figure 3: TSH Mean Concentration Values

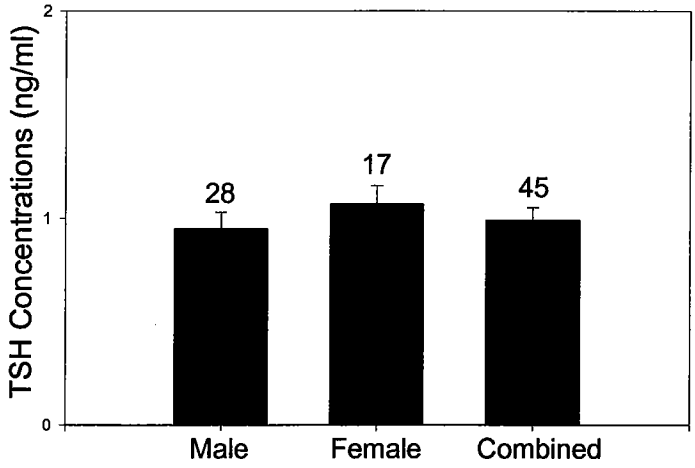


TABLE 1: T3, T4, and TSH concentration ranges, means, standard deviations, and standard error for female, male and combined populations.

	Range	Mean	Std Dev	SE
T3				
Female	28.7 ng/dl to 105.5 ng/dl	53.11	19.66	4.51
Male	13.0 ng/dl to 107.2 ng/dl.	58.62	23.42	4.21
Combined	13.0 ng/dl to 107.2 ng/dl.	56.53	22.03	3.11
T4				
Female	1.44µg/dl to 8.65 µg/dl	3.484	1.659	0.381
Male	< d.l.to 7.89 µg/dl.	3.632	1.819	0.338
Combined	< d.l. to 8.6 µg/dl.	3.573	1.741	0.251
TSH				
Female	0.63 ng/ml to 1.98 ng/ml	1.0701	0.3646	0.0884
Male	0.95 ng/ml to 2.77 ng/ml.	0.945	0.426	0.0805
Combined	0.6 ng/ml to 2.8 ng/ml.	0.9922	0.4043	0.0603

Perchlorate Residue Analysis

Fifty plasma samples were analyzed for perchlorate residues. One sample contained a trace amount of perchlorate but was below the quantifiable limits of the analytical procedure (2.5 ppb). All other samples were below the detection limit (1ppb).

The raccoon with trace amounts of perchlorate in plasma was caught near the fire station on the LHAAP. Potential food samples collected from the area around this particular capture event area were below detection limits.

Thyroid Hormone Concentration Comparisons

T3, T4, and TSH concentration values were compared to see if correlations existed between data sets. A significant negative correlation did not exist between T3 and TSH

(–0.182 (P=0.233)) or T4 and TSH (–0.024, (P=0.877)). T3 and T4 had a correlation coefficient of 0.45 (P=0.001). Figures 3-5 represent scatterplots of thyroid hormone concentrations.

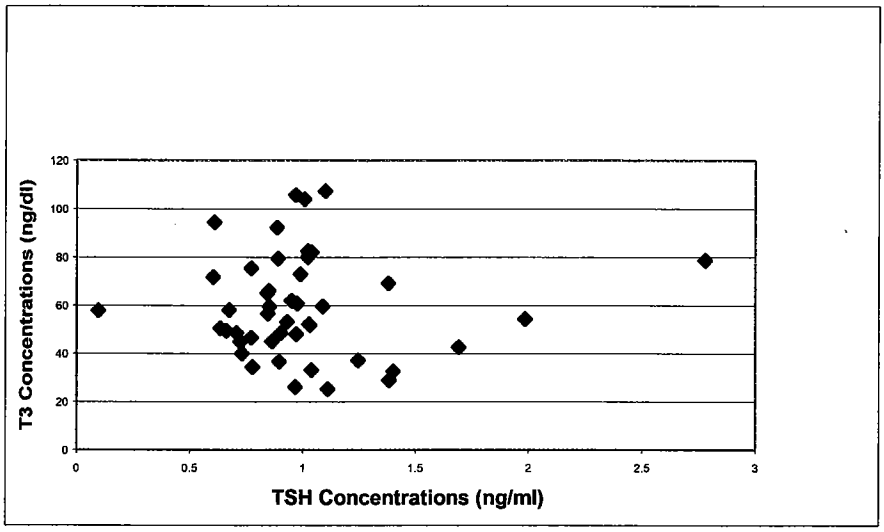


Figure 4: T3 vs. TSH Concentrations

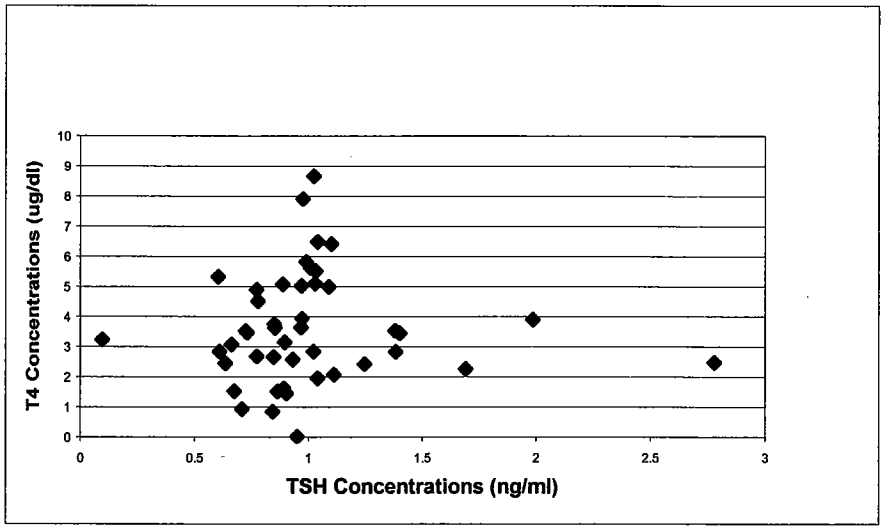


Figure 5: T4 vs. TSH Concentrations

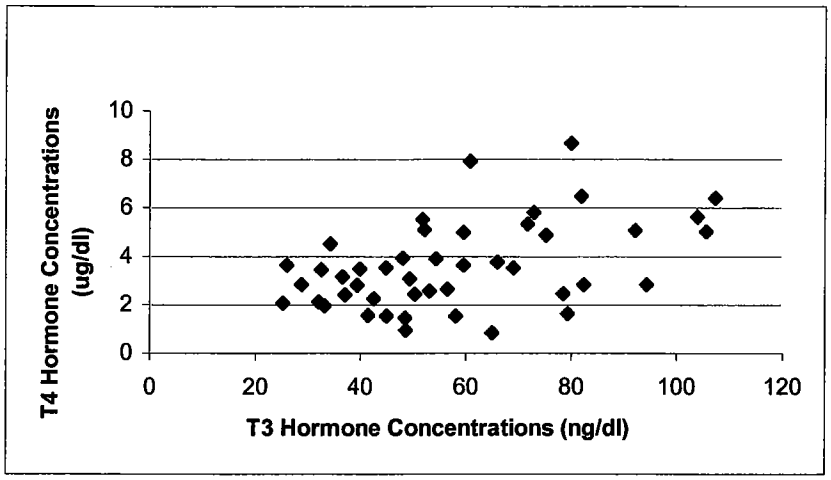


Figure 6: T3 vs. T4 Concentrations

Perchlorate Concentration of Potential Forage Items

Food items collected over the course of this study had varying levels of perchlorate concentrations (Table 2). Sample concentrations ranged from below detection limits to 5,557,000 ppb. All potential raccoon food items were found to have detectable concentrations of perchlorate in at least one sample with the exception of some plants. However, in general, plant matter had the highest perchlorate concentrations while fish had the lowest. The majority of insects (with the exception of four collections ranging from 881 to 48,341 ppb) were below detection limits. Water collected ranged from below detectable limits to 31,370 ppb.

TABLE 2: Perchlorate Concentrations (ppb) of potential forage items

Potential Forage Items	Site	N(N detects)	Detected Concentration (ppb)	Collection Date
Amphibians				
American Toad	Harrison Bayou Ponded Area	1(0)	Below detection levels	November 1999
Bullfrog	Harrison Bayou Ponded Area	2(0)	Below detection levels	November 1999
Bullfrog tapoles	INF Pond	3(3)	1130, 1277, 2567	November 1999
Chorus frog	INF Pond	1(1)	580	November 1999
Green Tree Frog	Harrison Bayou Discharge	1(1)	86	November 1999
Green Tree Frog	Harrison Bayou Ponded Area	1(1)	153	November 1999
Northern Cricket Frog	Harrison Bayou Ponded Area	1(0)	Below detection levels	November 1999
Northern Cricket Frog	Caddo Lake	1(0)	Below detection levels	November 1999
Fish				
Blackstripe top minnow	Goose Prairie Creek	2(1)	104	November 1999
Juvenile Sunfish	Harrison Bayou Ponded Area	1(1)	132	November 1999
Largemouth bass	Caddo Lake	5(0)	Below detection levels	November 1999
Largemouth bass	Caddo Lake	6(0)	Below detection levels	November 1999
Mosquitofish	Harrison Bayou Ponded Area	1(1)	206	November 1999
Mosquitofish	Goose Prairie Creek	2(2)	83, 131	November 1999
Notropis spp.	Harrison Bayou Ponded Area	2(1)	77	November 1999
Notropis spp.	Goose Prairie Creek	1(0)	Below detection levels	November 1999
Weed shiner	Harrison Bayou Ponded Area	3(1)	100	November 1999
Insects				
Bee	INF Pond	1(0)	Below detection levels	August 2001
Beetle	INF Pond	1(0)	Below detection levels	August 2001
Cricket	INF Pond	1(0)	Below detection levels	August 2001
Damselfly	INF Pond	1(0)	Below detection levels	August 2001
Damselfly larvae	INF Pond	3(3)	811, 1753, 2038	November 1999
Dragonfly	INF Pond	1(0)	Below detection levels	August 2001
Dragonfly	Burning Ground	1(1)	48341	August 2001
Exuviate	INF Pond	1(0)	Below detection levels	August 2001

Grasshopper	INF Pond	1(0)	Below detection levels	August 2001
Grasshopper	Burning Ground	1(0)	Below detection levels	August 2001
Orthroptera	INF Pond	1(0)	Below detection levels	August 2001
Wolf-Spider	Burning Ground	1(0)	Below detection levels	August 2001
Mammals				
Cotton Mouse	Harrison Bayou Discharge	2(1)	356	November 1999
Cotton Mouse	Building 25C	1(0)	Below detection levels	November 1999
Harvest Mouse	Harrison Bayou Discharge	1(0)	Below detection levels	November 1999
Harvest Mouse	Building 25C	2(2)	1120, 2328	November 1999
Southern short-tailed shrew	Building 25C	1(0)	Below detection levels	November 1999
Plants				
Alumina	INF Pond	1(0)	Below detection levels	May 2001
Berries	Building 25C	1(1)	593497	May 2001
Berries	INF Pond	2(1)	Very Contaminated	May 2001
Bullrush (above waterline)	INF Pond	4(4)	5975, 7211, 7816, 9387	November 1999
Bullrush (below waterline)	INF Pond	4(4)	2422, 3118, 4806, 7459	November 1999
Bullrush (roots)	INF Pond	2(2)	555, 1133	November 1999
Crabgrass (blades)	Building 25C	1(1)	5,557,000	October 2000
Crabgrass (blades)	Building 25C	1(1)	1,060,000	October 2000
Crabgrass (seeds)	Building 25C	1(1)	1,880,000	October 2000
Fern	Building 25C	2(2)	54, 2589	August 2001
Foxtail	Building 25C	2(2)	1865, 6346	May 2001
Goldenrod (leaves)	Building 25C	1(1)	1,030,000	October 2000
Goldenrod (seeds)	Building 25C	1(1)	184,000	October 2000
Goldrenrod (roots)	Building 25C	1(1)	140,000	October 2000
Goldrenrod (stems)	Building 25C	1(1)	6000	October 2000
Grass	Building 25C	2(1)	Below detection levels	August 2001
Grass	Building 25C	1(0)	Below detection levels	August 2001
Grass	INF Pond	1(1)	1846	May 2001
Grass	INF Pond	1(1)	9153	May 2001
Junucus	INF Pond	2(0)	Below detection levels	August 2001
Junucus	Building 25C	2(2)	76, 2350	August 2001

Junucus	INF Pond	1(0)	Below detection levels	August 2001
Saldigo sp. Leaves	Building 25C	2(2)	172079, 45918	May 2001
Saldigo sp. Stem	Building 25C	2(2)	18447, 8989	May 2001
Willow	Building 25C	2(2)	129, 19755	August 2001
Soil				
Sediment	Harrison Bayou Discharge	3(0)	Below detection levels	November 1999
Sediment	Goose Prairie Creek	3(1)	78	November 1999
Sediment	INF Pond	3(3)	12,185; 27,704; 35,630	November 1999
Sediment	Central Creek	3(0)	Below detection levels	November 1999
Soil	Building 25C	18(4)	50, 174, 249, 322	January 2000
Water				
Water	Harrison Bayou Discharge	3(1)	4	November 1999
Water	Goose Prairie Creek	3(3)	48, 81, 85	November 1999
Water	INF Pond	3(3)	30,776; 31,370; 31,438	November 1999
Water	Central Creek	3(1)	8	November 1999

November 1999 collection data taken from Smith et al, 2001; all other collection results from TIEHH data on file.

12.0 DISCUSSION

Results from this study did not indicate any appreciable perchlorate exposure among raccoons at the LHAAP or resulting effects on thyroid function. Perchlorate was detected at trace amounts in one raccoon plasma sample from a raccoon captured near in an area highly contaminated with perchlorate. Peak-blood levels after an oral intake are reported to occur after 3 hours and perchlorate's half-life in the rat is about 8 hours (Wolff, 1998). So, some exposure may occur and not be detected due to rapid clearance from the body or possibly due to the high limits of detections associated with our analytical procedure.

If perchlorate exposure resulted in thyroid hormone concentration alterations in raccoons, correlations between T3 and T4 and negative correlations between T3/T4, and TSH would be expected. For example, TSH levels would be high while corresponding T3 and T4 levels would be low. If perchlorate exposures among raccoons were sufficient to interfere with thyroid function, there would be a decrease in T3 and T4 output from the thyroid gland, and an increase in TSH secretion through the feedback mechanism that regulates the thyroid gland. Therefore, as T3 and T4 decrease TSH levels would increase resulting in a negative correlation. The Pearson Correlation Coefficients between TSH and T3 and T4 were –0.182 (P=0.233) and –0.024(P=0.877), respectively. The negative correlations between T3, T4 and TSH are not sufficiently strong enough to support the hypothesis that raccoon thyroid function was disrupted by perchlorate exposure at the LHAAP. The calculated Pearson coefficient for T3 and T4 was 0.45 (P=0.001) as expected.

Perchlorate dietary exposures were calculated to illustrate the range of perchlorate a raccoon inhabiting the LHAAP might ingest. Average daily intake and dietary composition (taken from US EPA, 1993) were compared with known perchlorate concentrations of raccoon food items collected at the LHAAP to calculate potential exposure. Summer season (scenario 1) exposures (based on 39% insects, 5% amphibian, 2% fish, 2% mammals, and 16% berries) illustrated perchlorate exposure ranges from 0 to 1,752 µg/day. Summer season (scenario 2) exposures (based on 37.9% fruit, 8.2% insects, 14.3% mammals, 4.4% amphibians, and 6.1% vegetation) illustrated exposure ranges from 0 to 462,570 µg/day. Winter season exposures (based on 12% insects, 7% amphibians, 2% fish, and 8% rodents) predicted up to 5021 µg/day. Based on these potential dietary exposure scenarios, raccoons in habiting the LHAAP do have the potential to consume considerable amounts of perchlorate at LHAAP.

This study encompassed trapping raccoons over a very large area, which included contaminated and uncontaminated areas; hence there was a spatial contamination gradient. This study was to include a radio telemetry and GIS analysis of the raccoon home ranges. The purpose of these procedures was to evaluate spatial exposure and responses. However, perchlorate residue was only found in one plasma sample at trace concentrations. Second, no negative correlation was seen among T3/T4 and TSH hormone levels. Since no exposure or effects were detected, we were unable to evaluate spatial exposure and responses. It is possible that the home ranges of the raccoons were much larger than contaminated areas. Therefore, there might not have been sufficient

exposure to see an effect on thyroid function. For example, a contaminated area might occur within their home range but not be in an area utilized with great frequency.

Perchlorate concentrations fluctuate temporally as well as spatially at this site due to remedial treatments and weather conditions. Since perchlorate may be excreted rapidly through urine, exposure, and certainly responses to perchlorate may be difficult to detect in raccoons at this site given our current analytical abilities.

Previous collections of small mammal tissue (e.g. harvest mice) from the LHAAP site contained perchlorate concentrations ranging from 589 to 2,170 ppb (Smith et al., 2001). Perhaps tissue collection from raccoons would provide a better indication of perchlorate exposure than plasma. However, the home ranges of small mammals undoubtedly play a role in their exposure to perchlorate and the results of the issue analysis.

Thyroid hormone concentrations are potentially good biomarkers for perchlorate contamination given that perchlorate competitively interferes with iodide accumulation in the thyroid (Wolf, 1998). However, at this point in time our results do not show an effect on thyroid hormone concentrations. This could be a result of variation in, or lack of, exposure and a lack of responsiveness of the raccoons to perchlorate. Even though the present results do not indicate thyroid hormone alterations among raccoons inhabiting the LHAAP, there could potentially be histological effects on thyroid tissue. Lower levels of perchlorate contamination that are not sufficient to effect hormone profiles could still affect the thyroid gland itself (e.g. through production hyperplastic cells and colloid tissue (Becker, et al, 1995). Thyroid histology would potentially be a more useful indicator of these low-level perchlorate effects.

It is likely that raccoons are exposed to perchlorate at relatively infrequent intervals and at low levels due to the intermittent perchlorate release at the site and their existence among a heterogeneous distribution of contaminants. Therefore, raccoons do not appear to be at risk of perchlorate exposure and resulting adverse effects at LHAAP. Sites with higher concentrations or a more constant source of perchlorate may represent a more tangible risk to raccoons.

13.0 REFERENCES

Becker, Kenneth, et al. Principles and Practice of Endocrinology and Metabolism. Philadelphia: Lippincott Company, 1995.

Beyer,, W.N., Connor, E.E., and Gerould, S. 1994. Estimates of soil ingestation by Wildlife. Journal of Wildlife Management. 58(2):375-382.

Norris, David, Vertebrate Endocrinology. San Diego: Academic Press. 1997.

Smith, Philip N., Theodorakis, D., Anderson, T., and Kendall, R. "Preliminary Assessment of Perchlorate in Ecological Receptors at the Longhorn Army Ammunition Plant (LHAAP) Karnak, Texas" Ecotoxicology 10 (2001) :305-313.

US EPA. 1993. Wildlife Exposure Factors Handbook. Office of Health and Environmental Assessment, Office of Research and Development. EPA/600/R-93/187.

Wolff, J. "Perchlorate and the Thyroid Gland" Pharmacology Reviews. 50 (1998): 89-105.

14.0 ACKNOWLEDGMENTS

Dr. Todd Anderson and graduate students, Jaclyn Canas and Sharon Williams, perfected sample extraction techniques and analyzed the raccoon plasma samples for perchlorate residues. Kim Gnadecki and Heidi Anderson assisted with raccoon data and sample collection.

Project No. T9700.6

A STUDY PROTOCOL

ENTITLED

Assessment of Perchlorate in Terrestrial Mammalian Receptors: Raccoons (*Procyon lotor*) and Opossums (*Didelphis virginiana*)

STUDY/PROTOCOL NUMBER: CAD-00-01

SPONSOR:

United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks AFB, PX 78235-5123

TESTING FACILITY:

Name/Address:

The Institute of Environmental & Human Health
Texas Tech University / Texas Tech University Health Sciences Center
Box 41163
Lubbock, Texas 79409-1163

Test Facility Management:

Dr. Ronald J. Kendall
Director, TIEHH

Study Director:

Dr. Philip N. Smith

PROPOSED EXPERIMENTAL
START DATE: April 1, 2000

1. DESCRIPTIVE STUDY TITLE:

Assessment of Perchlorate in Terrestrial Mammalian Receptors: Raccoons (*Procyon lotor*) and Opossums (*Didelphis virginiana*)

2. STUDY/PROTOCOL NUMBER: CAD-00-01

3. SPONSOR:

United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks AFB, PX 78235-5123
Department of Defense

4. TESTING FACILITY NAME & ADDRESS:

The Institute of Environmental & Human Health
Texas Tech University / Texas Tech University Health Sciences Center
Box 41163
Lubbock, Texas 79409-1163

5. PROPOSED EXPERIMENTAL START & TERMINATION DATES:

Start Date: April 1, 2000
Termination Date: December 31, 2001

6. KEY PERSONNEL:

Dr. Philip N. Smith, Study Director
Dr. Scott T. McMurry, Study Advisor
Ms. Ellen H. Roots, Research Associate
Ms. Kim Gniadecki, Research Assistant
Mr. Jody Wireman, Research Assistant
Mrs. Cindy McMurry, Research Assistant
Ms. Catherine M. Bens, Quality Assurance Manager
Mr. Ryan M. Bounds, Quality Assurance Officer
Dr. Ronald J. Kendall, Primary Investigator / Testing Facility Management

7. DATED SIGNATURES:

[signature]	3/8/00	Dr. Philip N. Smith Study Director
[signature]	3/9/00	Dr. Ronald J. Kendall Testing Facility Management
[signature]	3/9/00	Ms. Cathy Bens Quality Assurance Manager
[signature]	3/9/2000	Dr. Scott McMurry Study Advisor

8. REGULATORY COMPLIANCE STATEMENT

Quality Control and Quality Assurance

This study will be conducted in accordance with established Quality Assurance program guidelines and in compliance, where appropriate and possible, with Good Laboratory Practice Standards (40 CFR Part 160, August 17, 1989).

Document Control Statement

This document is considered proprietary to TIEHH and the Sponsor. Do not copy, quote or distribute. For access to this document or authority to release or distribute, please write to:

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9. STUDY OBJECTIVES / PURPOSE:

- To evaluate terrestrial mammalian exposure and effects of perchlorate through analysis of thyroid hormone profiles and determination of perchlorate concentrations in blood. We will attempt to document concurrent thyroid hormonal changes with perchlorate residues in blood. Raccoons and opossums afford the opportunity to collect large volumes of blood (in comparison to rodents) required for thyroid

hormone analysis and perchlorate anion analytical procedures.

- To delineate spatial exposure and responses among raccoons and opossums through radio-telemetry. Raccoons and opossums will be fitted with radio-collars and tracked throughout the study. Spatial data provided will assist in locating areas posing greatest risk, and may identify sources/areas of elevated perchlorate contamination. These data will document exposure in terrestrial mammalian receptors (or the lack thereof) in areas known to be contaminated with perchlorate.
- To evaluate the extent of developmental anomalies associated with perchlorate exposure. Previous research at Texas Tech University showed that perchlorate alters development and metamorphosis in amphibians (James Carr, data on file). As marsupials, opossums carry their embryos externally permitting a unique opportunity to observe embryonic development in a non-lethal manner. We will assess embryonic growth and development over time in relation to perchlorate exposure and maternal thyroid hormonal status.

10. TEST SITE PARAMETERS:

The Longhorn Army Ammunition Plant (LHAAP, approximately 8,000 acres), has been designated as a hazardous waste site on the US EPA National Priorities List. Perchlorate, a chemical heavily used at LHAAP, has been found in surface and ground water in many states throughout the Western United States. Ammonium perchlorate is used as an oxidizer in solid propellants, rockets, missiles, fireworks, and some munitions (Susarla et al., 1999), and readily dissociates into ammonium (NH_4^+) and perchlorate anion (ClO_4^-) in water. Perchlorate anion is extremely water-soluble and environmentally stable resulting in rapid movement through groundwater and surface water.

Numerous military activities at the LHAAP have resulted in the environmental dissemination of large quantities of perchlorate. Several locations at LHAAP have been associated with historical perchlorate handling, maintenance, or detonation including Building 25C, two separate burning grounds, a water treatment holding pond, and others. Ecologically sensitive wetland habitats including the Harrison Bayou drainage system, Goose Prairie Creek, Central Creek, and Caddo Lake surrounding these waste sites are at direct risk from perchlorate contamination through run-off, erosion, and groundwater movement. Recent field reconnaissance and subsequent analytical evaluation of ground and surface water, soil, and sediment samples taken from within the LHAAP boundaries indicated that perchlorate contamination at LHAAP is prevalent (TIEHH data on file) and potentially bioavailable to both aquatic and terrestrial organisms.

The test system boundaries will include all areas within the LHAAP, Karnack, Texas. The LHAAP is a non-functional army facility surrounded by an 8-ft tall security fence. It

is bordered on its north and east sides by Caddo Lake. All areas within the borders of LHAAP shall be considered as part of the test site.

11. JUSTIFICATION OF TEST SYSTEM

Raccoons assimilate approximately 0.0825 g water /g /day (US EPA, 1993). Perchlorate concentrations in surface water at LHAAP are quite variable, but concentrations in Harrison Bayou-fed ponds have been documented at 500 ppb (TIEHH data on file, 2000). At these concentrations, raccoons could easily ingest 124 – 289 ug/day (based on body weights of 3 – 7 kg). In addition to ingested water, raccoons may be exposed to perchlorate through food items and/or soil ingested with food or while grooming. Beyer et al. (1994) estimated that soil composed 9.4% of the diet of raccoons and opossums, and both species ingest plant matter that has been shown to accumulate perchlorate (Susarla et al., 1999). Raccoons and opossums are abundant on the LHAAP, and frequent many of the areas suspected to contain elevated concentrations of perchlorate (TIEHH data on file, 2000).

Raccoons and opossums were selected as sentinel species for this study based on potential for exposure and effects within the ecosystem surrounding LHAAP. Terrestrial mammalian receptors present on this site were evaluated for potential use as sentinel species based on characteristics such as abundance at LHAAP, exposure potential based on opportunistic feeding strategies in riparian zones, limited home range size (to be contained within LHAAP), social value as a furbearer, and previous use as sentinel species (Smith, 2000). Raccoons and opossums provided the best combination of desired sentinel species life history characteristics. Raccoons and opossums are inexorably linked with riparian environs, and therefore represent a potentially useful model for assessing perchlorate exposure and responses among terrestrial mammals. Previous research with raccoons has demonstrated home range fidelity to watersheds (Smith, 2000), similar to those contaminated with perchlorate at LHAAP. The reproductive strategies (external embryonic development) of opossums provide a unique opportunity to evaluate potential reproductive and/or developmental deficiencies resulting from perchlorate exposure. Other species considered as sentinel species were red (*Vulpes vulpes*) and gray foxes (*Urocyon cinereoargenteus*) and nutria (*Myocastor coypus*).

12. TEST ANIMALS (Where applicable provide number, body weight range, sex, source of supply, species, strain, sub-strain, and age of test system):

Species: Raccoons (*Procyon lotor*), Opossums (*Didelphis virginiana*)

Strain: Wild

Age: All ages, as encountered/trapped

Number: Approximately 70 raccoons, 70 adult opossums, 60 opossum embryos

Source: Collected/captured on or near LHAAP, Karnack, Texas

13. PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

All captured raccoons and adult opossums will be marked with uniquely numbered ear tags (placed in the outer lower portion of each ear) so that proper identification of study animals may be maintained throughout the study. In addition, a maximum of 40 total animals will be fitted with radio-transmitter collars so that area usage may by these raccoons may be defined. Collected embryos will be placed in uniquely identified containers.

14. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Raccoons and opossums will be collected from areas considered contaminated with perchlorate and other areas within LHAAP that have not produced perchorate contaminated samples. Concentrations of perchlorate and thyroid hormones in blood will be compared (regression techniques, see statistics section below) to detect elevated exposure and physiological responses. Additionally, ratios of thyroid stimulating hormone (TSH) to triiodothyronine (T3) and thyroxine (T4) will be examined to evaluate thyroid hormone imbalance. Elevated exposure or adverse responses will be related to geographic distribution of contaminants through radio-telemetry. Comparisons of blood perchlorate concentrations and thyroid hormone concentrations among animals occupying known-contaminated areas and designated "clean" areas (as they are identified) will be made to assess terrestrial mammalian exposure and response. Samples will be collected from within the defined boundaries of the study and reference site. Analytical and biochemical analysis will be performed in blind fashion, with researchers having no knowledge of where samples were collected to prevent analyst bias.

15. METHODS:

Trapping

Raccoons and opossums will be live-trapped in areas of concern and other areas at LHAAP. Traps will be placed in areas that show signs of raccoon or opossum activity (i.e., tracks, feces). Traps will be baited with a variety of baits which may include peanut butter, tuna fish, cat food, or other appropriate baits (TIEHH SOP ET 3-02-01). Raccoons and opossums will be anesthetized with a mixture of ketamine hydrochloride and xylazine hydrochloride injected with a syringe. While sedated, captured animals will be weighed (TIEHH SOP ET 3-14-01), sexed (male or female), aged (juvenile, adult) and

qualitatively examined for reproductive condition (pregnant, lactating, open, scrotal) and general health (good, fair, poor). Blood samples (see below) will be collected from each anesthetized animal (TIEHH SOP IN 3-08-01), placed in serum separator tubes and plasma collection tubes (containing EDTA) and centrifuged on an IEC MediSpin centrifuge at maximum speed for 12 minutes or until serum/plasma has been separated. Serum and plasma will be transferred to labeled tubes and frozen. Two Monel 1005-4 ear tags (brass-aluminum, style 893, National Tag & Band Co.) will be placed in the ears of each raccoon and opossum for identification. Prior to release, animals (those not selected for euthanization) will be monitored to insure recovery from anesthesia. Animals not selected for euthanasia will be released in their capture location. Trapping will be ongoing throughout the field portion of the study, and blood samples will be taken serially from recaptured animals.

Sample Collection

Blood (approximately 3-8ml) will be collected from each raccoon and opossum (TIEHH SOP IN 3-08-01) if possible. Serum and plasma samples will be kept frozen until analysis. Some animals inhabiting areas of concern or having altered thyroid hormone ratios (identified by perchlorate residues in serum or altered thyroid hormone profiles) will be euthanized and necropsied (TIEHH SOP IN 3-01-01). After sedation and blood sampling, a lethal overdose of sodium pentabarbitol will be injected intracardially on select individuals. The raccoons and opossums will be monitored until all respiration and cardiac function has ceased. Necropsies will be performed as described in (TIEHH SOP IN 3-01-01) and Smith (2000). Samples collected during necropsy (e.g. thyroid, liver, kidney, stomach, etc.) may be used for residue analysis and/or histology.

Opossum embryos will be examined for growth and development over time as dictated by recapture success (TIEHH SOP ET 3-01-01).

Telemetry

Following release, radio-collared raccoons and opossums will be located via radio-telemetry (TIEHH SOP ET 1-01-01) intermittently throughout the field portion of the study. Locations of radio-collared animals will be determined by direct contact or by triangulating detected radio-signal vector taken from at least three locations with known coordinates. Locations will be estimated using program LOCATE II. All spatial data will be collected in accordance with pending TIEHH SOPs on Differential Global Positioning System (DGPS).

Sample Analysis

Radioimmunoassays will be developed and optimized for thyroid stimulating hormone TSH, T3 and T4 levels in raccoons and opossums. SOPs will be developed at that time. Portions of whole blood will be transferred to the analytical lab (Todd Anderson) for

development of SOPs for detecting perchlorate in blood and analysis.

16. PROPOSED STATISTICAL METHODS:

Linear and/or logistic regression and Chi-square analysis will be used to examine the relationship between perchlorate exposure (as indicated through serum perchlorate concentrations) and thyroid hormone concentrations. Analysis of variance techniques may be used to evaluate differences in hormone (and possibly perchlorate concentrations) concentrations, embryonic development, etc., among areas considered contaminated and those designated as "clean."

17. REPORT CONTENT/RECORDS TO BE MAINTAINED:

Records to be maintained include field capture data, sample collection and handling logs, GPS coordinates of all captures and radio-telemetry data, analytical data, embryonic growth and development, and hormone analysis data.

Report content will include presentation of data, interpretation, and discussion of the following endpoints:

- Capture success
- Serum perchlorate concentrations
- Thyroid hormone concentrations
- Developmental anomalies
- Embryonic growth and development
- Spatial distribution (maps) of perchlorate exposure and thyroid hormone alterations

Interpretation of all data, including statistical results
Discussion of the relevance of findings
List of all SOPs used
List of all personnel

18. RECORDS TO BE MAINTAINED / LOCATION:

A final report will be delivered to the Sponsor as required by the Statement of Work and Cooperative Agreement. All data, documentation, records, protocol information, specimens shall be sent to the Sponsor, or designated delivery point, for final archive within six months of study completion. A copy of all data, the protocol and the final report shall be maintained by the testing facility.

19. QUALITY ASSURANCE:

The Quality Assurance Unit will inspect the study at intervals to insure the integrity of the study. Written records will be maintained indicating but not limited to the following:

date of inspection, study inspected, phase inspected, person conducting the inspection, findings and problems, recommended and taken action, and any scheduled reinspections. Any problems likely to effect study integrity shall be brought to the immediate attention of the Study Director. The Quality Assurance Unit will periodically submit written status reports on the study to management and the Study Director.

20. PROTOCOL CHANGES / REVISIONS:

All changes and/or revisions to the protocol, and the reasons therefore, shall be documented, signed and dated by the Study Director and maintained with the protocol and the Quality Assurance Unit.

21. REFERENCES:

Beyer, W.N., Connor, E.E., and Gerould, S. 1994. Estimates of soil ingestion by wildlife. Journal of Wildlife Management. 58(2):375-382.

Smith, P.N. 2000. Exposure and effects of polychlorinated biphenyls and metals in raccoons and selected rodents at the Paducah Gaseous Diffusion Plant in Western Kentucky. Doctoral Dissertation. Texas Tech University, Lubbock, Texas, USA. pp182.

Susarla, S., S. T. Bucchus, N. L. Wolfe, and S. C. McCutcheon. 1999. Phytotransformation of perchlorate and identification of metabolic products in *Myriophyllum aquaticum*. International Journal of Phytoremediation. 1:97-107.

US EPA. 1993. Wildlife Exposure Factors Handbook. Office of Health and Environmental Assessment, Offic of Research and Development. EPA/600/R-93/187.

A FINAL REPORT ENTITLED:
ENVIRONMENTAL MODELING

STUDY NUMBER:	MOD-01-01
SPONSOR:	Strategic Environmental Research and Development Program (SERDP) 1155 Herndon Parkway, Suite 900 Herndon, VA 20170
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Science Center Box 41163 Lubbock, TX 79409
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Science Center Box 41163 Lubbock, TX 79409
TEST SITE:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Science Center Box 41163 Lubbock, TX 79409
RESEARCH INITIATION:	January 1, 2001
RESEARCH COMPLETION:	December 31, 2001

GOOD LABORATORIES PRACTICES STATEMENT

This project, entitled "Environmental Modeling", was performed whenever possible in the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989.

Submitted By:

Kenneth Dixon, Ph.D

3/28/02
Date

1) **Sub- Project Title:**
Environmental Modeling

2) **Project Background:**
This research project will address perchlorate exposure to several animal species.

3) **Objective:**
The objectives of this sub-project are: 1) to develop mathematical models of the uptake, distribution and effects of the contaminants, 2) use computer simulation to predict long-term effects of the contaminants on wildlife populations, and 3) use the simulation results in a probabilistic risk assessment.

4) **Technical Approach:**
The modeling approach was to develop and apply mathematical models, either through modification of off-the-shelf models or *de novo* development, as appropriate, and parameterize the models using data from Phase III laboratory studies above.

Software tests are intended to challenge the application software and other parts of the overall system functionally and structurally. Functional testing demonstrates only that the system outputs appear to be correct. It does not allow an assessment of whether the software is actually performing according to specifications and requirements. A complete functional test of every combination of inputs may not be feasible except for very small programs. Functional testing is essentially a subset of structural testing.

Structural testing is designed to exercise all modules and branches of the software and their interrelationships with the hardware and peripheral devices. Structural testing is performed to ensure that all relevant functions in the software perform as intended.

Each of the testing types described below was conducted. Performing only one type of test will not prove that the system is working properly.

A. Normal Testing includes cases that test the functional and structural integrity of the computerized system. The input data for these test cases all fall within the range the user considers to be normal. Performing enough test cases can give a reasonable level of confidence that the system behaves as intended under normal conditions.

B. Boundary Testing is performed using values that force the system to discern whether the input is valid or invalid, or to make a decision as to which branch of the program to execute. Boundary test values are set at the edges (i.e., slightly below and above) of valid input ranges. Boundary testing does not mean making the computerized system "crash" or involuntarily stop.

C. Special Case Testing, also known as "exceptional case testing", documents the system's reactions to specific types of data or lack of data and is intended to ensure that the computerized system does not accept unsuitable data. These tests should be

designed to document what happens when values that are not included in the ranges defined in the specifications are entered. Use of test cases with no data entry in a field will assist in establishing software system defaults.

D. Parallel Testing is one of the most common types of tests performed by software developers. Parallel testing is performed by running two systems in parallel and comparing the outputs (e.g., two software application versions or software compared with a manual procedure). The comparison of the outputs from the same software release on different systems or different releases on the same system is part of parallel testing plans. Parallel testing can be a valuable tool when it is used in conjunction with other testing types for validation, or to train personnel to use a new computer system.

5) Project Accomplishments:

A. Develop groundwater and surface water models.

The modeling approach for the groundwater and surface water models have been developed using the Groundwater Modeling System (GMS) and the Watershed Modeling System (WMS). The COE has already generated similar models for total volatile organic carbon (VOC) concentrations at LHAAP using the GSSHA (Gridded Surface/Sub-surface Hydrologic Analysis) formulation of the hydrologic model CASC2D (Julien et al. 1995, Ogden 2000) in the WMS and FEMWATER from the GMS. To insure consistency across the models, the hydrologic parameters for LHAAP will be kept identical. To adjust the model for perchlorate we utilized sampling data from previous fieldwork to calibrate and run the model.

B. Develop PBTK models.

A Physiologically Based Toxicokinetic (PBTK) model was developed for perchlorate in the channel catfish (*Ictalurus punctatus*). The flow diagram (Figure 1) shows the compartments in the current model. Additional compartments can be added as the need is identified. The general equations used in the model were taken from a PBTK for lake trout (Lien et al. 2001). Physiological parameters, including partitioning coefficients, were derived from lab data for a 5-day 100ppb dosing study of channel catfish. Subsets of the data were used to calibrate the model. Additional exposure simulations were run using water concentrations measured at LHAAP (Smith et al. 2001).

The model was tested with each of the structural tests described above and passed each test.

Model Description

The model was developed and computer simulations were conducted on the uptake of perchlorate by channel catfish at the Longhorn Army Ammunition Plant (LHAAP) in Karnack, Texas. The model consists of two submodels: (1) a physiologically based

toxicokinetic (PBTK) model of the uptake and distribution of perchlorate in catfish body tissues for each individual in the population, and (2) a model of the thyroid hormone secretion and distribution as affected by the perchlorate concentration at the thyroid (Phase II). The model is stochastic in that it contains randomized concentrations of perchlorate in drinking water and partitioning coefficients within the range defined by field sampling and lab data respectively. These random variables provide the capability to conduct Monte Carlo simulations. The PBPK model includes compartments for gills, poorly perfused tissue (primarily white muscle and skin), richly perfused tissue (gut, GI tract, spleen, and gonads), kidney, liver, fat, and thyroid, (Figure 1).

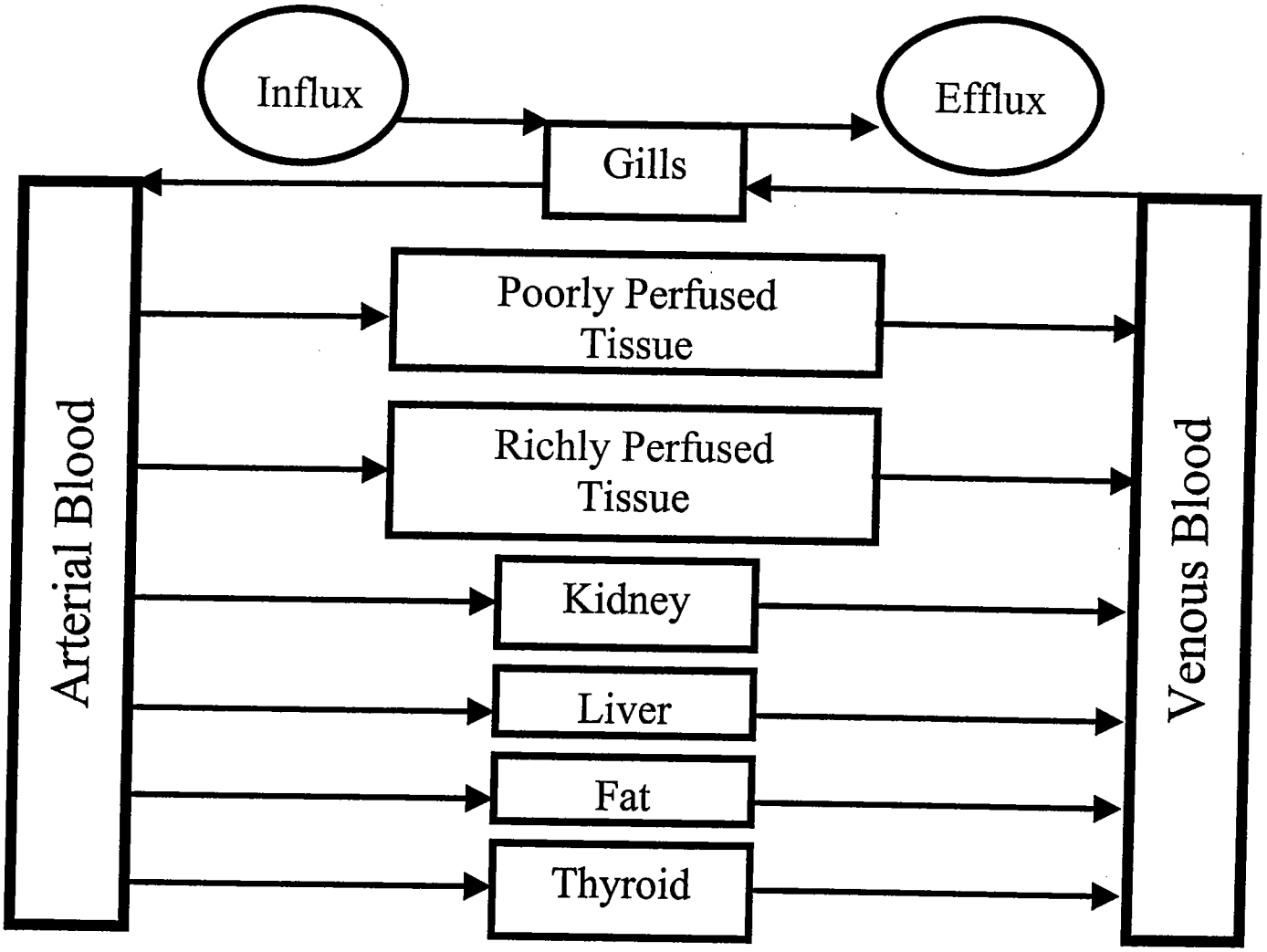

Figure 1 Flow Diagram of the PBTK model for perchlorate inhalation in fish.

Water intake (dose) is governed by the gill flux equation below :

$$F^G \quad k_X^G \; f_W C_W^{aff,G} \quad f_B C_B^{aff,G}$$

where
F^G = flux of perchlorate across the gills, mg#kg^{-1}#h^{-1}
k_X^G = exchange coefficient
f_W = ratio of free chemical in exposure water to total concentration
$C_W^{aff,G}$ = total concentration of perchlorate in exposure water, mg#kg^{-1}
f_B = ratio of free to total perchlorate in blood
$C_B^{aff,G}$ = concentration of perchlorate in the blood afferent to the gills, mg#kg^{-1}

The concentration of perchlorate in the blood afferent to the gills, also known as the venous blood, was calculated as follows:

$$C_B^{aff,G} \quad \frac{\{ \; C_B^{eff,i} * Q_B^i}{Q_B^G}$$

$C_B^{aff,G}$ = concentration of perchlorate in the blood afferent to the gills, mg#kg^{-1}
$C_B^{eff,i}$ = concentration of perchlorate in the blood efferent to the tissue compartment, mg#kg^{-1}
Q_B^i = blood flow rate from the tissue, L·h^{-1}
Q_B^G = total cardiac output, L·h^{-1}·kg

The concentration of perchlorate in blood efferent to each tissue compartment was calculated as follows:

$$C_B^{eff,i} \quad \frac{\clubsuit A^i \bullet}{\blacklozenge V^i \div \neq}$$

$C_B^{eff,i}$ = concentration of perchlorate in the blood efferent to the tissue compartment, mg#kg^{-1}
A^i = amount of perchlorate in the tissue, mg
V^i = volume the tissue compartment, kg

K_B^i = tissue/blood partitioning coefficient

The rate of change in perchlorate concentration for each tissue compartment is defined by the differential equation:

$$\frac{dA^i}{dt} \quad Q_B^i(C_B^{eff,G} \quad C_B^{eff,i})$$

$\frac{dA^i}{dt}$ = rate of change in perchlorate concentration in the tissue compartment, mg#kg^{-1}#h^{-1}
Q_B^i = blood flow rate from the tissue
$C_B^{eff,G}$ = concentration of perchlorate in the blood efferent to the gills, mg#kg^{-1}
$C_B^{eff,i}$ = concentration of perchlorate in the blood efferent to the tissue compartment, mg#kg^{-1}

Finally the concentration of perchlorate in the blood efferent to the gills was calculated as follows:

$$C_B^{eff,G} \quad C_B^{aff,G} \quad \frac{F^G}{Q_B^G}$$

$C_B^{eff,G}$ = concentration of perchlorate in the blood efferent to the gills, mg#kg^{-1}
$C_B^{aff,G}$ = concentration of perchlorate in the blood afferent to the gills, mg#kg^{-1}
F^G = flux of perchlorate across the gills, mg#kg^{-1}#h^{-1}
Q_B^G = total cardiac output, L·h^{-1}·kg

Calibration Run using lab data

To ensure the PBTK model accurately simulated the transport and fate of perchlorate in the channel catfish, it was necessary to calibrate the model using laboratory data. This was done by initially deriving the tissue/blood partitioning coefficients from measured concentrations in the respective tissue compartments. These coefficients were entered into the model as a mean +/- the standard deviation. The model was finally calibrated by adjusting the gill exchange coefficient, k_X^G. The model provided a good fit to the measured data as seen in table 1. In all graphs of the simulated output, red lines represent the population mean, green represents the upper 95% confidence limit, and blue represents the lower 95% confidence limit.

Compartment	Lab Mean (ppb)	Lab S.D. (ppb)	Simulated Mean (ppb)	Simulated S.D. (ppb)
Poorly Perfused Tissue	7261	1279	5900	1438
Richly Perfused Tissue	1326	507	1798	1026
Liver	126	174	166	74
Kidney	950	440	878	560
Fat	Not Measured	Not Measured	466	43
Thyroid Tissue	7222	1314	6581	3630

Table 1. Comparison of means and standard deviations for measured and simulated concentrations for a 5-day 100 ppb dose.

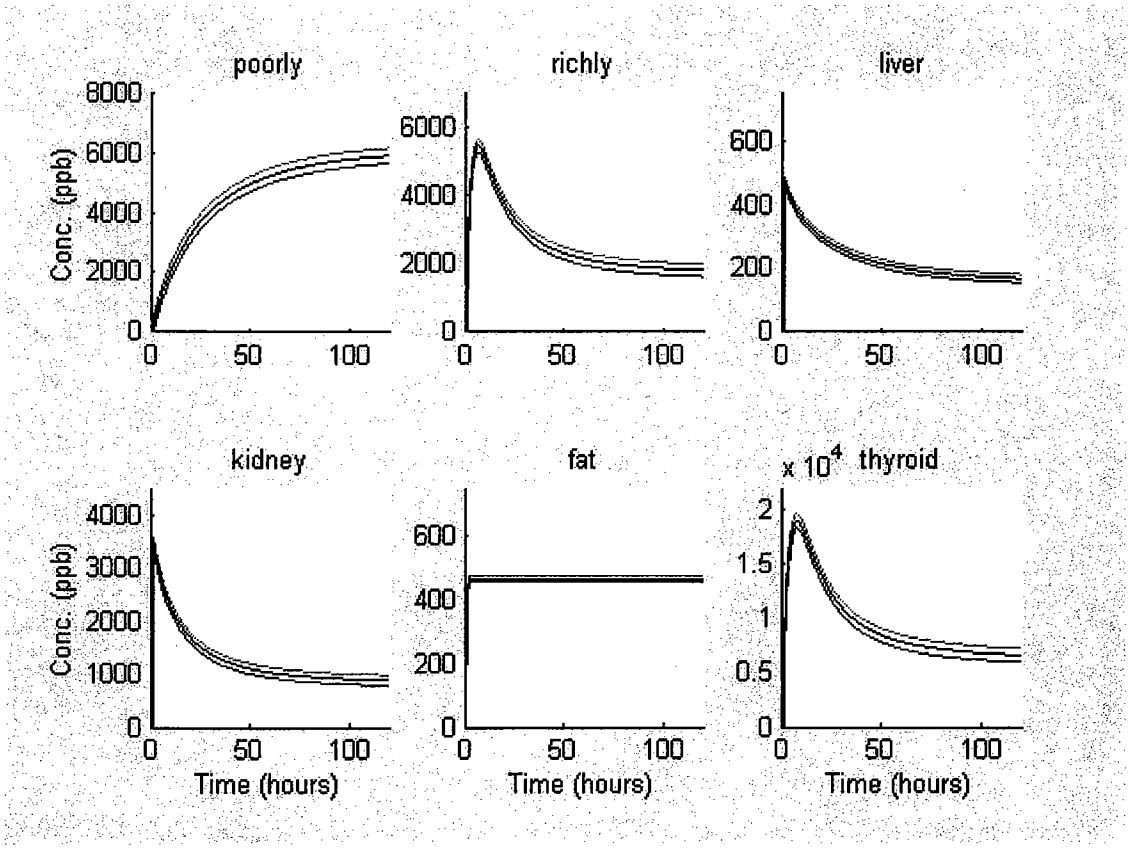

Figure 2. Simulated tissue concentrations over time in the channel catfish for a 5-day 100 ppb exposure.

Simulation Studies to Assess Remediation Alternatives

To simulate remediation alternatives, simulations were run for catfish populations inhabiting four drainages of LHAAP (Harrison Bayou, Goose Prairie Creek, Central Creek, and INF Pond). Only output from the Harrison Bayou (Figure 3) and INF Pond (Figure 4) areas are presented, as they represent the lowest and highest perchlorate concentrations, 4 ppb and 776 ppb respectively. The simulations for Harrison Bayou represent a site remediated to minimal perchlorate concentrations. Simulations for INF Pond represent little or no remediation of the site. It is important to note that the INF pond was used as a maximum-dose scenario, even though the high salinity in the pond precludes catfish from living there.

Results for Harrison Bayou

Simulation output included graphs of perchlorate concentrations in catfish organs and tissues (Figure 3) and the measured mean and standard deviation for each compartment (Table 2).

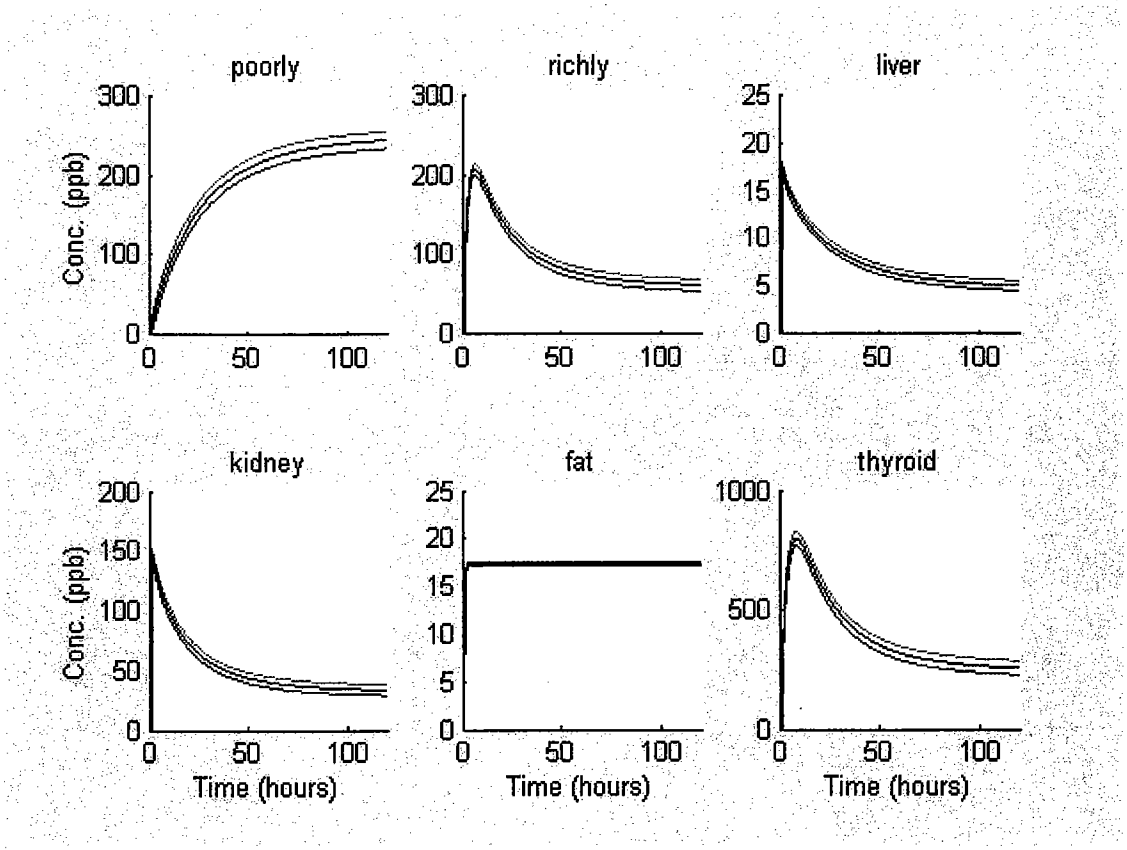

Figure 3. Simulated tissue concentrations over time in the channel catfish for a 5-day exposure at the Harrison Bayou site (4 ppb).

Results for INF Pond

Simulation output included graphs of perchlorate concentrations in catfish organs and tissues (Figure 4) and the measured mean and standard deviation for each compartment (Table 2).

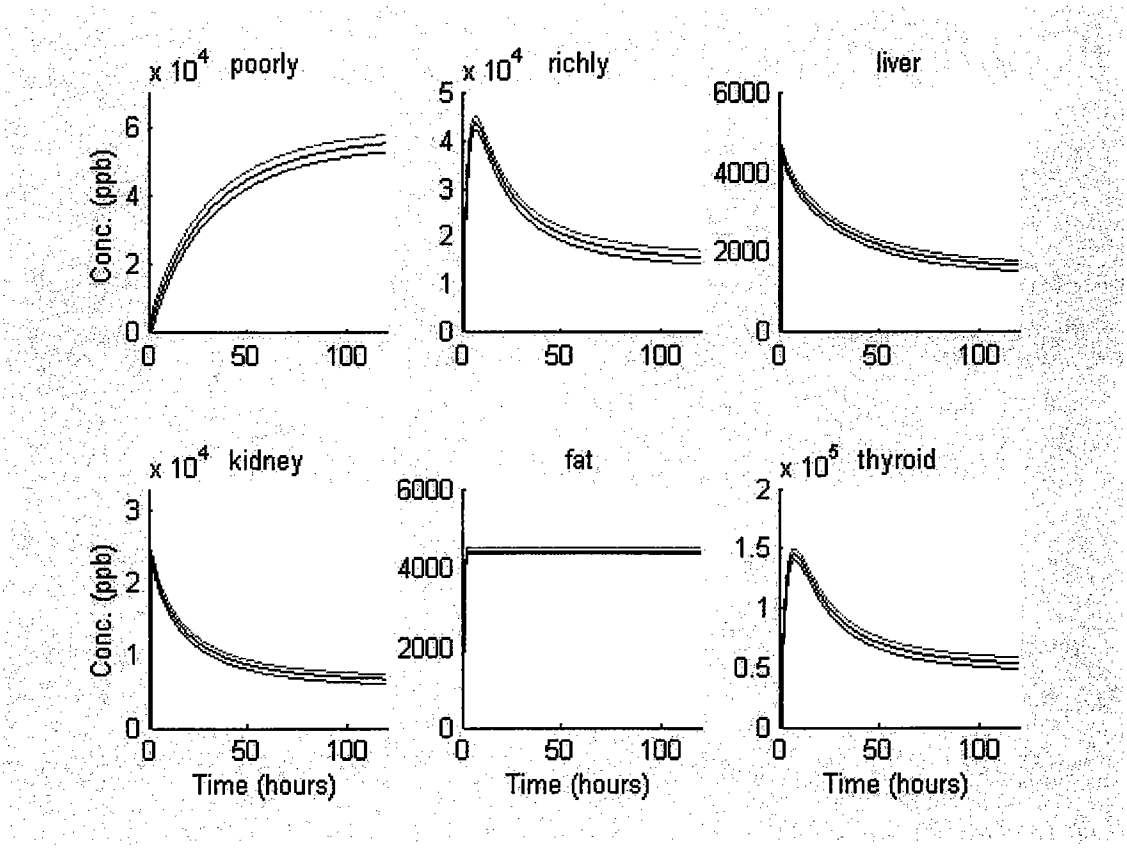

Figure 4. Simulated tissue concentrations over time in the channel catfish for a 5-day exposure at the INF Pond site (776 ppb).

Table 2. Comparison of Harrison Bayou and INF Pond simulations.

	LHAAP Study Site			
	Harrison Bayou (4 ppb)		*INF Pond (776 ppb)*	
Compartment	**Mean (ppb)**	**S.D. (ppb)**	**Mean (ppb)**	**S.D. (ppb)**
Poorly Perfused	244	60	55,137	14,013
Richly Perfused	61	42	15,533	8,103
Liver	5	3	1,638	722

Kidney	33	26	6,637	3,861
Fat	17	2	4,426	406
Thyroid	254	164	52,790	27,346

Thyroid Submodel

This model was discussed in detail in the Phase II report, where it was attached to a raccoon PBTK model to show the effect of perchlorate levels on hormone secretion. This same model has been linked to the PBTK for the catfish. Currently data is being collected on hormone secretion levels that will allow us to adjust the thyroid model specifically for the catfish.

C. Fleshing File

A "fleshing file" has been developed which allows for the linkage and visualization of the various models that have been and currently are being developed. We can now utilize the simulated surface water concentrations as the dose for our PBTK instead of relying on a constant single value. An example simulation converts the output generated from the GMS/WMS packages into a 3-dimensional matrix of concentrations, where the position within the matrix represents the coordinate location of that particular concentration. This plume was then degraded by 5% each hour for 96 hours to provide a dynamic system. To define the dose received by the catfish, a point was randomly generated within the original extent of the plume, lake dimensions in this example, for each hour with the concentration at that point being the dose. These doses are then entered into the PBTK model generating similar output as seen above. By programming in a trilinear interpolation subroutine within the model we were able to generate the location point to the fourth decimal place. This approach saves large amounts of computing time since only 96 points are generated instead of the 172,800,000 that would be necessary with the trilinear function that comes with MATLAB® for a 30x30x2 matrix over 96 hours. This corresponds to a processing time of seconds instead of days. A movie of the fish movement overlaid on the degrading plume is also generated allowing for easy visual recognition and explanation. In all of the plots that follow, asterisks represent the position of the fish at the previous and current time step to allow for directional orientation. The numeric value over one of the asterisks is the dose received at that point for that hour. The plume slices represent the top and bottom of a lake with individual concentrations determined by the trilinear interpolation subroutine discussed earlier.

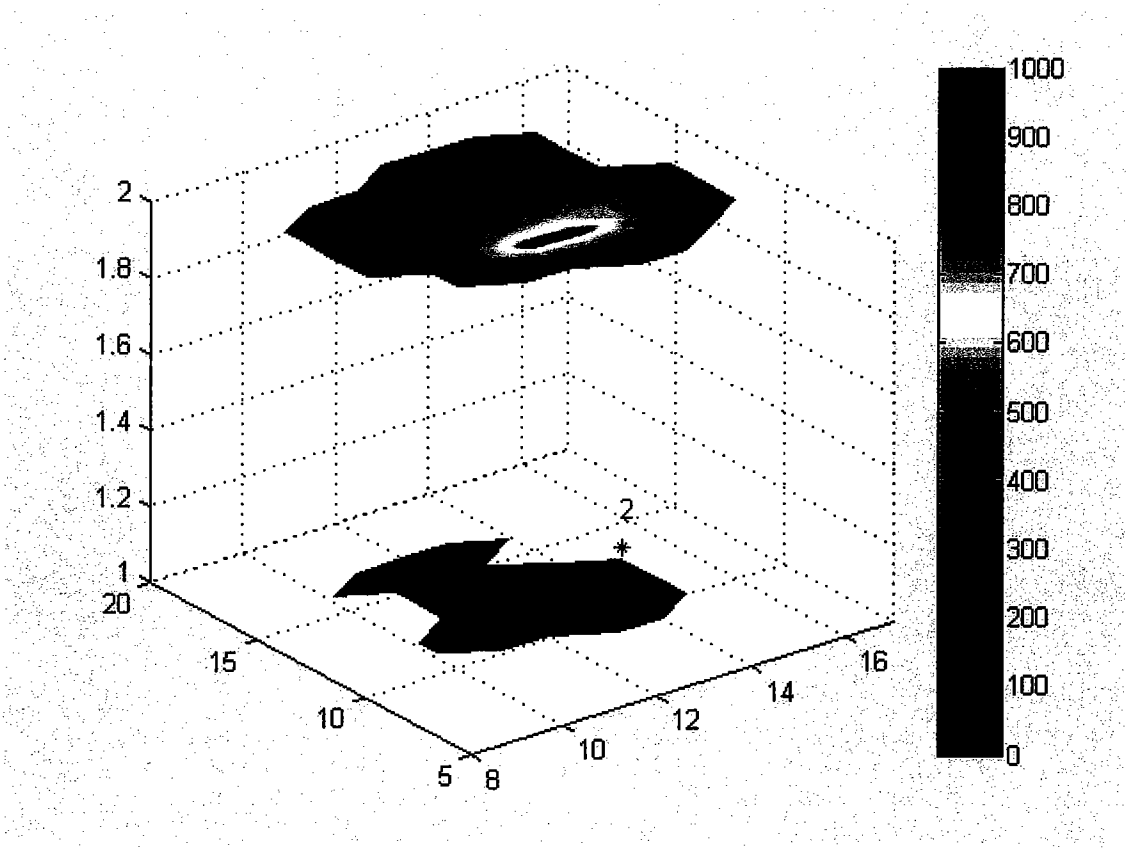

Figure 5. Fleshing file output for hour 1.

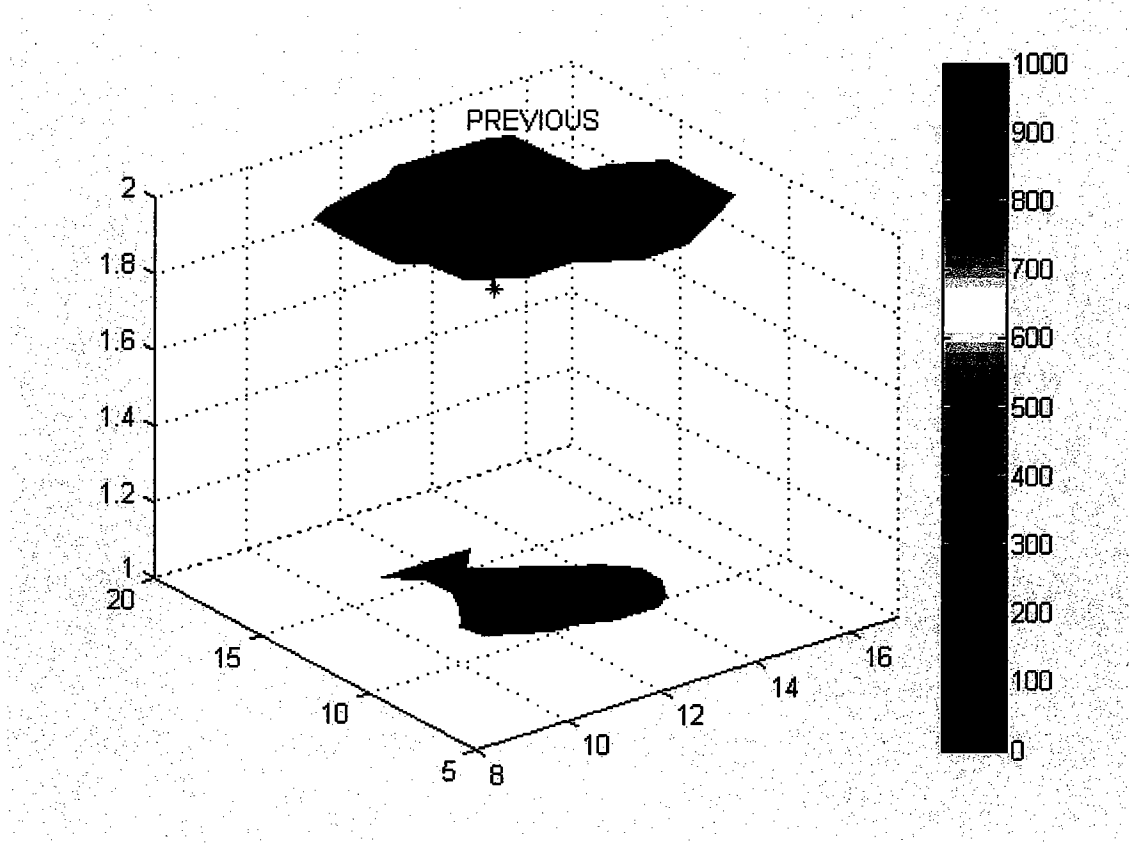

Figure 6. Fleshing file output for hour 48.

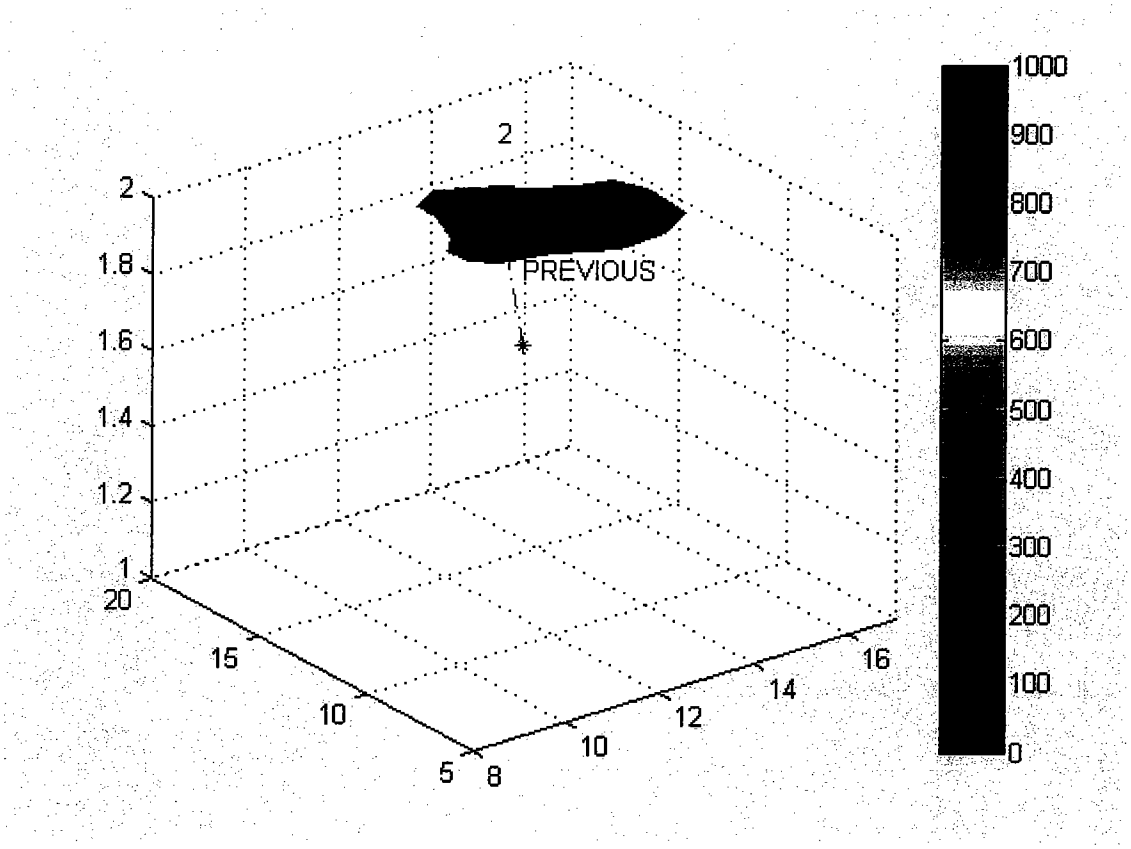

Figure 7. Fleshing file output for hour 96.

Discussion and Conclusion

There were significantly higher perchlorate concentrations predicted in all model catfish organ and tissue compartments for the INF Pond area, compared with the Harrison Bayou drainage. Until we receive new data on hormone levels in channel catfish we cannot reliably simulate the impacts on the thyroid tissue. Once we receive the appropriate computer files for the groundwater and surfacewater models we can rapidly calibrate them with field data we have already collected. While the fleshing file is written for a simplistic system, the underlying theories and methods hold constant for more complex ones, allowing for rapid modification for almost any scenario. Through the use of the fleshing file we will be able to more accurately simulate the actual dose received by the channel catfish and the subsequent response, allowing for more informed decision-making.

6) Literature Cited

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Smith, P.N., C. W. Theodorakis, T. A. Anderson, and R. J Kendall. 2001. Preliminary assessment of perchlorate in ecological receptors at the Longhorn Army Ammunition Plant (LHAAP), Karnack, Texas. Ecotoxicology. 10:305-313.

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7) Associated Personnel:

Eric Albers – graduate student

A FINAL REPORT

ENTITLED

FISH AND AMPHIBIANS AS AQUATIC SENTINELS FOR PECHLORATE EXPOSURES AND EFFECTS AT THE LONGHORN ARMY AMMUNITION PLANT, JEFFERSON COUNTY, TEXAS

STUDY NUMBER:	AQUA-01-01
SPONSOR:	Strategic Environmental Research and Development Program (SERDP) 1155 Herndon Parkway, Suite 900 Herndon, Virginia 20170
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, Texas 79409-1163
TEST SITE:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, Texas 79409-1163
ANALYTICAL TEST SITE:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
RESEARCH INITIATION:	02/12/2001
RESEARCH COMPLETION:	09/31/2001

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List of Tables and Figures

GOOD LABORATORIES PRACTICES STATEMENT

This project, entitled "Fish and amphibians as aquatic sentinels for perchlorate exposures and effects at the Longhorn Army Ammunition Plant, Jefferson County, Texas", was performed whenever possible in the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989

Submitted By:

Christopher Theodorakis, Ph.D

3/28/02
Date

QUALITY ASSURANCE STATEMENT

This study was conducted under the Institute of Environmental and Human Health's Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Any changes in protocol and SOPs were documented in writing and signed by the study director.
The Quality Assurance Officer verbally notified the Study Director of all findings at the time of the inspection. Written audit reports were also submitted to the Study Director and Test Facility Management. Audits were performed for the following phases of the project:

Auditable Research Phase / Activity	Audit Dates		Date written report submitted to Study Director	Date written report submitted to Management
	Start	End		
Final Report and Raw Data Review	2/25/02	3/06/02	3/19/02	3/19/02

Submitted By:

Ryan Bounds
Quality Assurance Manager

Date 3/20/02

1. DESCRIPTIVE STUDY TITLE

Fish and amphibians as aquatic sentinels for perchlorate exposures and effects at the Longhorn Army Ammunition Plant, Jefferson County, Texas

2. STUDY NUMBER

AQUA-01-1

3. SPONSOR

Strategic Environmental Research
and Development Program (SERDP)
1155 Herndon Parkway, Suite 900
Herndon, Virginia 20170

4. TESTING FACILITY NAME AND ADDRESS

The Institute of Environmental and Human Health
Texas Tech University
Box 41163
Lubbock, Texas 79409-1163

5. PROPOSED EXPERIMENTAL START & TERMINATION DATES

Start Date: 02/12/2001
Termination Date: 9/31/2001

6. KEY PERSONNEL

Dr. Christopher Theodorakis, Study Director
Dr. Todd Anderson, Analytical Chemist
Dr. Ronald Kendall, Testing Facilities Management/Principal Investigator
Ryan Bounds, Quality Assurance Manager
Emilia Cruz-Li, Technician
Carrie Bradford, Technician

7. STUDY SUMMARY

Sunfish were collected from different sites at LHAAP to evaluate the effects of pechlorate on thyroid follicle cell height and follicle hyperplasia (over stimulated cell division). No significant differences were found between individuals collected from reference and contaminated sites. Follicular epithelial hyperplasia was noted only for white crappie collected from a contaminated site (Harrison Bayou).

Mosquitofish were collected from different sites at LHAAP in order to determine the possible effects of perchlorate on their reproductive parameters such as fecundity and number of abnormal embryos. The data did not provide evidence of differential reproductive success among sites in a pattern that would be concordant with patterns of perchlorate exposure.

8. STUDY OBJECTIVES / PURPOSE

-To determine the extent of exposures of ammonium perchlorate to fish and effects on thyroid function.
-To determine relative body burdens of perchlorate in fishes and amphibians of various trophic levels in surface waters of LHAAP.

9. TEST MATERIALS

Test Chemical name: Perchlorate anion
CAS number: 7790-98-9
Characterization: Determination of concentration in environmental samples
Source: Wastewater treatment effluent discharge

10. JUSTIFICATION OF TEST SYSTEM

Preliminary surveys of LHAAP have revealed that measurable levels of ammonium perchlorate have been found in surface waters of aquatic systems (i.e., streams, ponds, and bayous) within and adjacent to LHAAP. However, the uptake, bioaccumulation, and tissue distribution of perchlorate in fish and amphibians has not been studied to date. Information on the acute and chronic toxic effects of perchlorate in fish are little known. Previous studies by our group have addressed acute toxicity of perchlorate on zebrafish (*Brachydanio rerio*) and African clawed frogs (*Xenopus laevis*). In addition, studies in scientific literature indicate that thyroid homeostasis is important in reproductive functions such as gonadal development, growth, and embryonic development. Because perchlorate is a thyroid disrupter (Miranda et al., 1996; Manzon and Youson, 1997), indices of thyroid damage or disruption may be appropriate biomarkers for perchlorate exposures. Because zebrafish and African frogs are non-native species, additional studies using native fish (e.g., mosquitofish) and amphibians (e.g., spring peepers) were accomplished through collection and analysis of individuals.

11. TEST ANIMALS (where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test system)

Species: Western mosquitofish (*Gambusia affinis*), Minnows (*Notropis spp.*) and any other fish species deemed suitable as determined by abundance of specific aspects of their biology
Strain: Wild animals
Age: Various

Number: Maximum of 300 per species
Source: Captures from natural waters at LHAAP

12. PROCEDURE FOR IDENTIFYING THE TEST SYSTEM

The test system was natural waters within LHAAP. TTU and private contractors have identified contaminated sites in previous surveys. Reference sites, selected based on proximity to LHAAP and similarity to LHAAP water bodies, were not found to contain detectible levels of perchlorate. Each sampling location was labeled with its whole name or a 4-letter abbreviation. Four contaminated sites have been identified. Their names (and 4 letter abbreviations) were Harrison Bayou "catfish pond" (HBCP), Harrison Bayou upstream (HBUP), Goose Prairie Creek (GPCK), holding pond (HOLP). There have also been four reference sites identified: Central Creek (CENC), Star Pond (STAR), Haggerty Creek (HAGC) and Karnack Creek (KARC). Any other sites that were added were referenced either by their full name of the 4 letter abbreviation, determined as follow: the names of ponds, creeks, etc, were abbreviated with the 1st three letters of the name followed by P (pond), C (creek), R (river), L (lake), or B (bayou) (e.g., Jim's Bayou = JIMB, Caddo Lake = CADL). If the name of the creek, lake, etc has only 4 letters, this was used in place of a 4-letter abbreviation (Star Pond = STAR). If the water body consists of 2 or more words, the last letter of the abbreviation indicated type of water body (P=pond, etc.), and the other letters represented at least the first letter of each word of a compound name, and additional letters in the name were added to total 4 letters, if needed (e.g., Little Cypress Bayou may be abbreviated LCYB or LICB, provided the same abbreviation is used for all samples; East Fork Poplar Creek would be abbreviated EFPC). All samples taken from the same water body within 100 meters were counted as a single sample. If 2 or more samples were taken at intervals greater than 100 meters, or if a series of samples were taken from a stretch of lake, creek, etc, that is more than 100 meters long; the samples was suffixed with numbers (e.g., HAGC-1, HAGC-2, etc.). If a pond, lake, creek did not have a name associated with it, it was labeled with a letter, e.g., Pond A, Pond B, Lake A, Creek A, Creek B, Creek C, etc. The 1st 3 letters of the abbreviation was PND (pond), CRK (creek), LKE (Lake), BYU (bayou) or RIV (river), followed by A, B, C, etc. (e.g., Pond A = PNDA). All names and abbreviations were recorded on data sheets and sample tracking forms and/or in the field notebook for future reference.

13. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL

Fish were collected from sites of known perchlorate contamination and at least 1-2 reference sites. As many individuals as can be collected were taken from each site, up to 20 individuals per site. Individuals were temporarily held in a bucket until processed. Different buckets were used for each site or else buckets were washed between sampling sites. Before taking previously used buckets into the field or if washing between sampling sites was required, buckets were washed with detergent and tap water, and rinsed with tap water or other water known to be uncontaminated with perchlorate or other toxicants. The bucket was then rinsed with tap water (or other water) 3 times. If

mud, algae, or other residues remained on the inside of the bucket, it was scrubbed off during washing (a different brush was used for each sampling site). Reference sites were chosen so as to be as similar as possible to the contaminated site(s) in terms of habitat structure and stream characteristics. The area of pond/lake (m^2) or length of stream or lakeshore (m) sampled at each site was as similar as possible between sampling sites. The length of time animals were held in buckets was minimized, and was as similar as possible for all sites. The same species were collected from contaminated and reference sites. For thyroid analysis, the fish collected were as similar in size as possible between sites. Fish were weighed prior to processing and weights were recorded on sampling form.

14. METHODS

14.1 Test System acquisition, quarantine, acclimation

14.1.1 Water Sampling

At each location where fish were captured, 60 ml of water was taken for perchlorate analysis, according to SOP AQ-3-03. Water was collected before collection of fish, if at all possible. Water samples were collected at either end of the section of the stream from which fish were collected, plus at least one sample in between these two points. Samples were taken at least 10 m apart, unless the section of stream to be sampled is less than 10 m long. Water samples were collected in precleaned glass vials (Wheaton), and were collected from just under the water surface. Water samples were stored away from direct sunlight and excessive heat (> 50° C). Before samples were taken, the pH, dissolved oxygen, conductivity, and temperature were measured according to SOP IN-2-01 and recorded on TIEHH Form 181.

14.1.2 Fish Collection

Fish were collected with backpack electroshocker set at a current of 2-4 amps and a frequency of 30-60 cps; or they were collected with a seine, dip net or baited traps as described in SOP AQ-3-05 "Fish and Amphibian Field Collection Methods". Traps were placed at least 1 m apart and checked at most every 24 hours. Traps were anchored to a non-movable object on the shore with highly visible nylon twine or firmly attached to a highly visible floating buoy and anchored to the bottom. Traps were regularly spaced, or concentrated in habitats where target species were known to occur. Because mosquitofish and topminnows are not susceptible to electroshocking, they were collected by seining. The seine was at least 20' long and 4' deep, with a mesh of ¼" or smaller. Any captured fish was placed in plastic buckets with aeration until processing. A different bucket was used for each site, or the buckets were cleaned as

described in section 13. Fish were identified as belonging to a particular family and labeled accordingly (see SOP IN-1-06). If it was not identified to family, it was labeled as an unidentified fish (see SOP IN-1-06).

14.1.3 Amphibian Collection

Larval or aquatic amphibians were collected with dip nets and seines according to SOP AQ-3-05. Adult amphibians were collected by hand, with dipnets, or with pitfall traps.

14.2 Test Material Application

Rates/concentrations: Concentration determined by laboratory analysis.
Frequency: Perchlorate was discharged into Harrison Bayou by Radian Corporation 4 times per week, whenever there was water flowing in Harrison Bayou. When there was no water flowing in Harrison Bayou, the perchlorate-laden water was discharged into the holding pond. The mechanism of entry of perchlorate into Goose Prairie Creek is unknown, but is suspected to occur during rain events or continually from groundwater seepage.
Route/Method of Application: Ingestion of absorbtion of perchlorate from water and natural food items.
Justification for Exposure Route: The animals were exposed to perchlorate in water and food items in their natural environment.
Exposure Verification: Water samples were collected for determination of perchlorate concentrations wherever biota samples were collected.

14.3 Test System Observation

At every location from which water samples were taken, the following environmental parameters were evaluated: water temperature, pH, salinity, dissolved oxygen, and conductivity.

14.4 Animal Sacrifice and Sample Collections

14.4.1 Data Recording and Sample Labeling

Prior to processing any samples, they were given a unique ID number and species and weight were recorded on sample collection/dissection forms, as well as tissues collected and method of preservation. Fish were weighed on a portable balance to the nearest gram (if the fish weighs 10 grams or more) of the nearest 1/10 gram if it weighs less than l0 grams. Prior to use, the scale was calibrated according to SOP IN-4-01, "Field Scale Operations and Maintenance" and calibration recorded on TIEHH Form 60, or in bound field notebook.
According to the SOP IN-3-02, the minimum information to be recorded

on labels was the project number and unique ID. The unique ID contained enough information to be able to identify the species and tissue. The unique ID included the 4-letter abbreviation of the species (see SOP IN-1-06) followed by the sample number. For example, YLBH-1 is the unique ID for yellow bullhead #1. Sample numbers was assigned in the order in which they were processed. If a sample was a composite, then the letters COMP was added to the ID. For example: YLBH-COMP-1 was a composite sample of yellow bullhead catfish. If a sample was divided into subsamples, a suffix consisting of a decimal point and a number was assigned. For example, if YLBH-1 was divided into 2 subsamples, the IDs for these subsamples were YLBH-1.1 and YLBH-1.2. If the composite YLBH-COMP-1 was divided into subsamples, the subsamples were labeled YLBH-COMP-1.1 and YLBH-COMP-1.2. If a fish was dissected into constituent organs, the label of each sample was suffixed with a 2-letter abbreviation designating the organ, as follows:

Tissue	Abbreviation
Fillet	FL
Head	HD
Gills	GL
Whole blood	BD
Plasma	PL
Liver	LV
Gonad	GD
GI tract	GI

For example, YLBH-1-BD was the whole blood sample from fish YLBH-1, MOSQ-COMP-1-PL was composite sample #1 of mosquitofish plasma, and LMBA-1.2-BD was subsample #2 from the blood of largemouth bass #1. Labels for whole bodies did not contain a suffix (e.g., YLBH-1 implies this sample is a yellow bullhead whole body).

14.4.1 Perchlorate analysis

Various species of different trophic levels were collected for perchlorate body burden analysis. Fish collected were anesthetized with an overdose of MS222 (0.5 g/L) and frozen in liquid nitrogen for perchlorate analysis. Individual fish were wrapped in aluminum foil and labeled prior to freezing or chilling. Smaller fish were consolidated into composite samples. Composite samples were placed into Ziploc freezer bags and stored on ice until transport back to the laboratory. At least 5 g of tissue is needed for perchlorate analysis; therefore fish smaller than this were composited.

After transport to the laboratory, samples were stored in the freezer (temperature -20° C or colder) until analysis. The samples were then analyzed for perchlorate according to SOP AC-2-15 "Extraction and

Cleanup of Tissue Samples to be Analyzed for Perchlorate".
Water samples also were transported back to the laboratory for perchlorate analysis. Once in the laboratory, they were stored in a refrigerator (4° C) until analysis. They were then analyzed for perchlorate according to SOP AC-2-11 "Analysis of Perchlorate by IC".

14.4.2 Thyroid Hormone Histology

Juvenile sunfish were collected via backpack electroshocker from Harrison Bayou and reference sites. All fish were then transported in water from the collection site to a makeshift laboratory onsite at LHAAP. Fish were anesthetized in MS222, and the heads were severed and preserved in Bouin's fixative for analysis.
Preserved samples were decalcified by incubation in Bouin's fixative, followed by soaking in distilled water until most of the fixative was removed. They were then dehydrated in ethanol and embedded in paraffin blocks, and sectioned using a Tissue-Tek III embedding platform. The sections were mounted onto microscope slides, stained with Hematoxylin and Eosin stain, and examined under a light microscope fitted with a micrometer.
For each fish, ten follicular epithelial cells were measured from each of 10 follicles (100 cells total). Cell height was recorded to the nearest micron. Also, follicle cell hyperplasia (as evidenced by multiple layers of follicle epithelium, rather than just one layer) was also noted.

14.4.3 Reproductive Assessment

These experiments consisted of 2 collections, one in 5/2001 and one in 7/2001. For the May collections, adult female mosquitofish were collected from 2 sites that receive perchlorate - Goose Prairie Creek at Karnack Avenue and Goose Prairie Creek at Crocket Avenue - and 3 reference sites: Star Pond, Central Creek, and Big Cypress Bayou. Females were preserved in ethanol and the fecundity was determined as number of embryos/body length, because it has been found that the number of embryos in this species is directly correlated to the size of the female. Also the percent gravid females (# females with egg or embryos/total number of females x 100) and number of embryos with abnormal development were also recorded. For the July collections, fish were collected from Goose Prairie Creek and Central Creek. The analyses were performed as above.

14.5 Endpoint Analysis

Analysis of samples for perchlorate were done according to SOP AC-2-11 "Analysis of Perchlorate by IC".
Analysis of tissues for histology were done according to SOP AQ-2-03 "General

Histological Processing of Thyroid Follicles in Small Fish".

14.6 Statistical Methods

All data were checked for normality using the Shapiro-Wilk *W* test. Homogeneity of variances was checked using (Bartlett's or Lavine's test). Comparisons between sites were accomplished by Analysis of Variance (ANOVA) for multiple mean comparisons. Correlation coefficients were used to determine if residue levels correlate with biomarkers, reproductive, and/or population data.

15. PROTOCOL CHANGES/REVISIONS

No protocol changes and/or revisions were necessary to complete this study.

16. RESULTS

16.1. Effects of perchlorate on field-collected fish as reflected by thyroid histology

At each location, several species were collected. These included warmouth sunfish (*Lepomis gulosus*), bluegill sunfish (*L. macrochirus*) and redear sunfish (*L. microlophus*). However, the species composition varied from site to site, so that the only species that were collected in sufficient numbers for analysis from both Harrison Bayou and a reference site were warmouth sunfish and redear sunfish. No significant differences were found between individuals collected from Harrison Bayou vs. the reference site (Haggerty Creek), for either species (Table 1). The only evidence of follicular epithelial hyperplasia was noted for white crappie collected from Harrison Bayou (2 of the 3 fish collected exhibited hyperplasia in at least one follicle examined), but unfortunately no individuals could be found at the reference site. The height of the follicles of crappie from Harrison Bayou was greater than that from any other species, but it is not known if this is due to interspecific susceptibility to perchlorate or if it is due to differences in normal physiology between species.

16.2. Effects of perchlorate on reproductive parameters in mosquitofish

16.2.1 May collections

There were no significant differences in the fecundity of the females from Central Creek and either site from Goose Prairie Creek, nor between Star Pond and Big Cypress Bayou, but both Star Pond and Big Cypress Bayou had statistically significant lower fecundity than the other three sites (Table 2, ANOVA, $p<0.05$). There were no significant differences any sites in terms of % gravid females, and no evidence of embryo malformation was evident.

16.2.2 July collections

Mosquitofish were collected from Goose Prairie Creek at the Crocket Avenue Bridge and from Central Creek. The mean (± SD) fecundity for these locations was 0.95 ± 0.57 and 0.34 ± 0.30, respectively, and these differences were statistically significant ($p<0.05$, t-test). There were 100% gravid females at Goose Prairie Creek and 73 % gravid females at Central Creek.

Table 1. Mean (± SD) thyroid follicle epithelial cell height (microns) of fish collected from LHAAP).

	Location	
Species	Haggerty Creek	Harrison Bayou
Warmouth sunfish	2.77 ± 0.55	3.20 ± 0.93
Redear sunfish	3.57 ± 0.98	3.03 ± 0.68
Bluegill	ND	2.90 ± 0.64
White Crappie	ND	4.90 ± 0.52

Table 2. Reproductive parameters of mosquitofish collected from various locations at LHAAP.

Site	Mean (± SD) Fecundity[a]	% Gravid Females[b]
Goose Prairie Crockett	1.31 ± 0.56	100
Goose Prairie Karnack	1.26 ± 0.45	100
Central Creek	1.25 ± 0.70	86
Star Pond	0.59 ± 0.14	100
Big Cypress Bayou	0.68 ± 0.33	93

[a]Number of embryos/length of the female.
[b](Number of females containing eggs or embryos/total number of females in sample) x 100.

17. DISCUSSION

There seems to be a lot of variability between sites in terms of fecundity, which can introduce environmental "noise" and obscure any effects of perchlorate. The data did not provide evidence of differential reproductive success among sites in a pattern that would be concordant with patterns of perchlorate exposure. This assay does not seem to be a sensitive indicator of effects of perchlorate exposure at LHAAP.

18. STUDY RECORDS AND ARCHIVE

Study Records will be maintained at The Institute of Environmental and Human Health (TIEHH) Archive for a minimum of one year after study completion date.

19. REFERENCES

Manzon RG and Youson JH. 1997. Immunocytochemical and morphometric study of TSH, PRL, GH, and ACTH cells in Bufo arenarum larvae with inhibited thyroid function. Gen. Comp. Endocrinol. 98: 166-176.

Miranda, LA, Paz, DA, Dezi, RE and Pisano, A. 1996. Immunocytochemical and morphometric study of TSH, PRL, GH, and ACTH cells in Bufo arenarum larvae with inhibited thyroid function. Gen. Comp. Endocrinol. 98: 166-176.

20. APPENDICES

Study Protocol

Project No. T9700.8

A STUDY PROTOCOL

ENTITLED

FISH AND AMPHIBIANS AS AQUATIC SENTINELS FOR PECHLORATE EXPOSURES AND EFFECTS AT THE LONGHORN ARMY AMMUNITION PLANT, JEFFERSON COUNTY, TEXAS

STUDY/PROTOCOL NUMBER: AQUA-01-1

SPONSOR:

US Air Force
AFIERA/RSRE
2513 Kennedy Cir
Brooks AFB, TX 7235-5123

ADMINISTRATOR: TIEHH

TESTING FACILITY:

Name/Address:

The Institute of Environmental and Human Health
Texas Tech University/Texas Tech University Health Sciences Center
P.O. Box 41163
Lubbock, TX 79409-41163

Test Facility Management: Dr. Ronald Kendall

Study Director: Dr. Christopher Theodorakis

PROPOSED EXPERIMENTAL
START DATE: 2/12/01

1. **DESCRIPTIVE STUDY TITLE:**
Fish as Aquatic Sentinels for Contaminant Exposures and Effects at the Longhorn Army Ammunition Plant, Jefferson County, Texas

2. **STUDY NUMBER:** AQUA-01-1

3. **SPONSOR:**

United States Air Force
IERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

4. **TESTING FACILITY NAME & ADDRESS:**

Texas Tech University
The Institute of Environmental and Human Health
PO Box 41163
Lubbock TX 79406-1163

5. **PROPOSED EXPERIMENTAL START & TERMINATION DATES:**
Start Date: 2/12/01
Termination Date: 9/31/01

6. **KEY PERSONNEL:**
Dr. Christopher Theodorakis, Study Director
Dr. Todd Anderson, Analytical Chemist
Dr. Ronald Kendall, Testing Facilities Management/Principal Investigator
Ryan Bounds, Quality Assurance Manager
Carrie Hendrickson, Technician

7. **DATED SIGNATURES:**

2/9/01 Dr. Christopher Theodorakis
Study Director

2/13/00 Dr. Ronald Kendall
Testing Facility
Management/PI

2/13/01 Ryan Bounds
Quality Assurance Manager

2-13-01 Dr. Todd Anderson
Analytical Chemist

8. REGULATORY COMPLIANCE STATEMENT

Quality Control and Quality Assurance

This study will be conducted in accordance with established Quality Assurance program guidelines and in compliance, where appropriate and possible, with Good Laboratory Practice Standards (40 CFR Part 160, August 17, 1989).

Document Control Statement

This document is considered proprietary to and the Sponsor. Do not copy, quote or distribute. For access to this document or authority to release or distribute, please write to:
Dr. Ronald J. Kendall
Texas Tech University
The Institute of Environmental and Human Health
Lubbock, TX 79409-1163 USA

9. STUDY OBJECTIVES / PURPOSE:

-To determine the extent of exposures of ammonium perchlorate to fish and effects on thyroid function.
-To determine relative body burdens of perchlorate in fishes and amphibians of various trophic levels in surface waters of LHAAP.

10. TEST MATERIALS:

Test Chemical name: Perchlorate anion
CAS number: 7790-98-9
Characterization: Determination of concentration in environmental samples.
Source: Wastewater treatment effluent discharge.

11. JUSTIFICATION OF TEST SYSTEM

Preliminary surveys of LHAAP have revealed that measurable levels of ammonium perchlorate have been found in surface waters of aquatic systems (i.e., streams, ponds, and bayous) within and adjacent to LHAAP. However, the uptake, bioaccumulation, and

tissue distribution of perchlorate in fish and amphibians has not been studied to date. Information on the acute and chronic toxic effects of perchlorate in fish are little known. Previous studies by our group have addressed acute toxicity of perchlorate on zebrafish (*Brachydanio rerio*) and African clawed frogs (*Xenopus laevis*). In addition, studies in scientific literature indicate that thyroid homeostasis is important in reproductive functions such as gonadal development, growth, and embryonic development.

Because perchlorate is a thyroid disrupter, indices of thyroid damage or disruption may be appropriate biomarkers for perchlorate exposures. Because zebrafish and African frogs are non-native species, additional studies using native fish (e.g., mosquitofish) and amphibians (e.g., spring peepers) will be accomplished through collection and analysis of individuals.

12. TEST ANIMALS (Where applicable provide number, body weight range, sex, source of supply, species, strain, sub-strain, and age of test system):

Species: Species: Western mosquitofish (*Gambusia affinis*), Minnows (*Notropis spp.*) and any other fish or amphibian species deemed suitable as determined by abundance of specific aspects of their biology (to be determined on-site).

Strain: Wild animals.

Age: Various

Number: Maximum of 300 per species.

Source: Captures from natural waters at LHAAP.

13. PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

The test system will consist of natural waters within LHAAP. TTU and private contractors have identified contaminated sites in previous surveys. Reference sites were selected based on proximity to LHAAP and similarity to LHAAP water bodies, and were not found to contain detectible levels of perchlorate. Each sampling location will be labeled with its whole name or a 4-letter abbreviation. To date, four contaminated sites have been identified. Their names (and 4 letter abbreviations) are Harrison Bayou "catfish pond" (HBCP), Harrison Bayou upstream (HBUP), Goose Prairie Creek (GPCK), holding pond (HOLP). There have also been four reference sites identified: Central Creek (CENC), Star Pond (STAR), Haggerty Creek (HAGC) and Karnack Creek (KARC). Any other sites that may be added will be referenced either by their full name of the 4 letter abbreviation, determined as follow: the names of ponds, creeks, etc, will be

abbreviated with the 1st three letters of the name followed by P (pond), C (creek), R (river), L (lake), or B (bayou) (e.g., Jim's Bayou = JIMB, Caddo Lake = CADL). If the name of the creek, lake, etc has only 4 letters, this may be used in place of a 4-letter abbreviation (Star Pond = STAR). If the water body consists of 2 or more words, the last letter of the abbreviation will indicate type of water body (P=pond, etc.), and the other letters will at least represent the first letter of each word of a compound name, and additional letters in the name may be added to total 4 letters, if needed (e.g., Little Cypress Bayou may be abbreviated LCYB or LICB, provided the same abbreviation is used for all samples; East Fork Poplar Creek would be abbreviated EFPC). All samples taken from the same water body within 100 meters may be counted as a single sample. If 2 or more samples are taken at intervals greater than 100 meters, or if a series of samples are taken from a stretch of lake, creek, etc, that is more than 100 meters long; the samples will be suffixed with numbers (e.g., HAGC-1, HAGC-2, etc.). If a pond, lake, creek does not have a name associated with it, it will be labeled with a letter, e.g., Pond A, Pond B, Lake A, Creek A, Creek B, Creek C, etc. The 1st 3 letters of the abbreviation will be PND (pond), CRK (creek), LKE (Lake), BYU (bayou) or RIV (river), followed by A, B, C, etc. (e.g., Pond A = PNDA). All names and abbreviations must be recorded on data sheets and sample tracking forms and/or in the field notebook for future reference.

14. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Fish will be collected from sites of known perchlorate contamination and at least 1-2 reference sites. As many individuals as can be collected will be taken from each site, up to 20 individuals per site. Individuals will be temporarily held in a bucket until processed. Different buckets will be used for each site, if possible, or else buckets will be washed between sampling sites. Before taking previously used buckets into the field or if washing between sampling sites is required, buckets will be washed with detergent and tap water, and rinsed with tap water or other water known to be uncontaminated with perchlorate or other toxicants. The bucket will then be rinsed with tap water (or other water) 3 times. If mud, algae, or other residues remain on the inside of the bucket, it will be scrubbed off during washing (a different brush will be used for each sampling site). Reference sites will be chosen so as to be as similar as possible to the contaminated site(s) in terms of habitat structure and stream characteristics. The area of pond/lake (m^2) or length of stream or lakeshore (m) sampled at each site should be as similar as possible between sampling sites. The length of time animals are held in buckets should be minimized, and should be as similar as possible for all sites. The same species will be collected from contaminated and reference sites. For thyroid analysis, the fish collected should be as similar in size as possible between sites. Fish will be weighed prior to processing and weight will be recorded on sampling form.

15. METHODS:

15.1 Test System acquisition, quarantine, acclimation

Water Sampling

At each location where fish are captured, 60 ml of water will also be taken for perchlorate analysis, according to SOP AQ-3-03. Water should be collected before collection of fish, if at all possible. Water samples should be collected at either end of the section of the stream from which fish were collected, plus at least one sample in between these two points. Samples should be taken at least 10 m apart, unless the section of stream to be sampled is less than 10 m long. Water samples will be collected in precleaned glass vials (Wheaton), and will be collected from just under the water surface. Water samples should be stored away from direct sunlight and excessive heat (> 50° C). Before samples are taken, the pH, dissolved oxygen, conductivity, and temperature should be measured according to SOP IN-2-01 and recorded on TIEHH Form 181.

Fish Collection

Fish may be collected with backpack electroshocker set at a current of 2-4 amps and a frequency of 30-60 cps; or they may be collected with a seine, dip net or baited traps as described in SOP AQ-3-05 "Fish and Amphibian Field Collection Methods". Traps should be placed at least 1 m. apart and checked at most every 24 hours. Traps should be anchored to a non-movable object on the shore with highly visible nylon twine or firmly attached to a highly visible floating buoy and anchored to the bottom. Placement of traps may be regularly spaced, or concentrated in habitats where target species are known to occur. Because mosquitofish and topminnows are not susceptible to electroshocking, they should be collected by seining. The seine should be at least 20' long and 4' deep, with a mesh of ¼" or smaller. Smaller seines may be used if seining smaller water bodies. If the water is too deep for backpack shocking, shocking may be done by boat. In smaller water bodies, the backpack shocker generator and power supply may be disconnected from the backpack frame and secured on the boat. In larger bodies of water, a boat electroshocking device may be used. Any captured fish will be placed in plastic buckets with aeration until processing. A different bucket will be used for each site, or the buckets will be cleaned as described in section 14. If any fish is captured that is not readily identifiable in the field, one or more specimens will be anesthetized in 0.5 g/L MS222 (SOP AQ-1-03) and preserved in 10% neutral buffered formalin (approx. 10 ml for each g body weight) and transported back. If it can be identified as belonging to a particular family, it will be labeled as an unidentified member of that family (see SOP IN-1-06). If it cannot be identified to family, it will be labeled as an unidentified fish (see SOP IN-1-06).

Amphibian Collection
Larval or aquatic amphibians will be collected with dip nets and seines according to SOP AQ-3-05. Adult amphibians will be collected by hand, with dipnets, or with pitfall traps.

15.2 Test Material Application

Rates/concentrations: Concentration determined by laboratory analysis.

Frequency: Perchlorate will be discharged into Harrison Bayou by Radian Corporation 4 times per week, whenever there is water flowing in Harrison Bayou. When there is no water flowing in Harrison Bayou, the perchlorate-laden water is discharged into the holding pond. The mechanism of entry of perchlorate into Goose Prairie Creek is unknown, but is suspected to occur during rain events or continually from groundwater seepage.

Route/Method of Application: Ingestion of absorbtion of perchlorate from water and natural food items.

Justification for Exposure Route: The animals are exposed to perchlorate in water and food items in their natural environment.

Exposure Verification: Water samples will be collected for determination of perchlorate concentrations wherever biota samples are collected.

15.3 Test System Observation

At every location from which water samples are taken, the following environmental parameters will be evaluated: water temperature, pH, salinity, dissolved oxygen, and conductivity.

15.4 Animal Sacrifice and Sample Collections

Data Recording and Sample Labeling

Prior to processing any samples, they will be given a unique ID number and species and weight will be recorded on sample collection/dissection forms, as well as tissues collected and method of preservation. Fish length is also an optional parameter than can be recorded. Fish weight will be measured on a portable balance to the nearest gram (if the fish weighs 10 grams or more) of the nearest 1/10 gram if it weighs less than l0 grams.

Prior to use, the scale needs to be calibrated according to IN-4-01, "Field Scale Operations and Maintenance" and calibration should be recorded on TIEHH Form 60, or in bound field notebook.

According to the SOP IN-3-02, the minimum information to be recorded on labels is the project number and unique ID. The unique ID will contain enough information to be able to identify the species and tissue. Date of collection, species, sex and tissue may also be included on the label. Pre-printed labels may be used, but if they are used on samples to be frozen or chilled on ice, the project number and unique ID must also be printed on the container with indelible ink.

The unique ID will include the 4-letter abbreviation of the species (see SOP IN-1-06) followed by the sample number. For example, YLBH-1 is the unique ID for yellow bullhead #1. Sample numbers will be assigned in the order in which they are processed. If a sample is a composite, then the letters COMP will be added to the ID. For example: YLBH-COMP-1 is a composite sample of yellow bullhead catfish. If a sample is divided into subsamples, a suffix consisting of a decimal point and a number will be assigned. For example, if YLBH-1 and is divided into 2 subsamples, the IDs for these subsamples would be YLBH-1.1 and YLBH-1.2. If the composite YLBH-COMP-1 is divided into subsamples, the subsamples would be labeled YLBH-COMP-1.1 and YLBH-COMP-1.2. If a fish is dissected into constituent organs, the label of each sample will be suffixed with a 2-letter abbreviation designating the organ, as follows:

Tissue	Abbreviation
Fillet	FL
Head	HD
Gills	GL
Whole blood	BD
Plasma	PL
Liver	LV
Gonad	GD
GI tract	GI

For example, YLBH-1-BD would be the whole blood sample from fish YLBH-1, MOSQ-COMP-1-PL would be composite sample #1 of mosquitofish plasma, and LMBA-1.2-BD would be subsample #2 from the blood of largemouth bass #1. Labels for whole bodies do not need to contain a suffix (e.g., YLBH-1 implies this sample is a yellow bullhead whole body).

Perchlorate Analysis:

Various species of different trophic levels will be collected for perchlorate body burden analysis. Fish collected will be anesthetized with an overdose of MS222 (0.5 g/L) and frozen in liquid nitrogen for perchlorate analysis. Alternatively, fish may be chilled on wet ice until transport back to the laboratory. Fish should not chilled on wet ice for more than 5 days before processing or being frozen. Individual fish will be wrapped in aluminum foil and labeled prior to freezing or chilling. Smaller fish may also be consolidated into composite samples. Composite samples will be placed into Ziploc freezer bags and stored on ice until transport back to the laboratory. At least 5 g of tissue is needed for perchlorate analysis; fish smaller than this must be composited.

After transport to the laboratory, samples will be stored in the freezer (temperature -20° C or colder) until analysis. The samples will then be analyzed for perchlorate according to SOP AC-2-15 "Extraction and Cleanup of Tissue Samples to be Analyzed for Perchlorate".

Water samples also are transported back to the laboratory for perchlorate analysis. Once in the laboratory, they will be stored in a refrigerator (4° C) until analysis. They will then be analyzed for perchlorate according to SOP AC-2-11 "Analysis of Perchlorate by IC".

Thyroid Hormone Analysis:

If any individuals that are large enough to collect at least 50 µl of blood (i.e., fish typically with 30 g total mass or more), they will be anesthetized in 0.5 g/L MS222 until they lose the righting reflex and do not respond to physical stimuli, but before ventilation of the gills completely ceases. The species, weight, date and location of collection will be recorded on fish dissection or multiple dissection/collection forms or in a bound notebook. Blood will then be collected with EDTA-treated vaccutainer tubes or EDTA-treated syringes according to SOP AQ-3-06 "Collection of Blood from Fish". If possible, blood from smaller fish can be collected by anesthetizing the fish (as above), severing the caudal peduncle, and collecting blood from the caudal vein using heparinized microhematocrit tubes. The minimum amount of blood necessary for thyroid analysis is 50 µl. Blood from smaller fish may be composited to achieve this amount. The minimum number of blood samples required to obtain a sample over 50 µl will comprise each composite. Blood will then immediately be transferred to a microcentrifuge tube and placed on ice. Within 10 min after collection, the blood will be centrifuged at 3000-5000 rpm for 5 min. Plasma will then be siphoned off of the pelleted blood cells using a Pasteur pipette or micropipette, transferred to a 2 ml cryogenic storage vial suitable for

liquid nitrogen immersion, and will be immediately frozen in liquid nitrogen. The plasma will be transported back to the laboratory in liquid nitrogen and stored in a freezer (-70° C or colder) until analysis.

Thyroid hormones analysis for small fish (e.g., mosquitofish, minnows) requires whole-body analysis. For this analysis, whole fish are wrapped in aluminum foil or placed in cryogenic vials (described above) and frozen in liquid nitrogen. Upon arrival at the laboratory, the samples will be stored in a freezer (-70° C or colder) until analysis. This analysis requires at least 2 g of tissue. If fish are smaller than 2 g, they will be composited. The minimum number of fish required to obtain a sample is 1- 2 g in each composite.

Thyroid Hormone Histology

Fish that are smaller than 5 g will be preserved whole in Bouin's fixative. Fish will first be anesthetized in 0.5 g/L MS 222, then immersed in Bouin's fixative. If Bouin's is not available, fish may be preserved in 10% buffered formalin (10 ml Bouin's or Formalin for each gram of fish). For

If they are preserved in Bouin's, the samples need to be chilled at 0-4° C (e.g., on ice or in a refrigerator). Within 48 hours of initial preservation, the Bouin's then needs to be poured into a waste container, and the sample jar/vial and the samples need to be rinsed with distilled or RO water (rinsates are to be poured into the waste jar). The samples then need to be preserved in 70% ethanol (ambient temperature) until transport back to the laboratory (at least 10 ml ethanol per gram sample). No such treatment is necessary if the samples are preserved in formalin (i.e., they may be stored in formalin at ambient temperatures indefinitely). Any waste materials need to be transported back to the laboratory and disposed of in a manner consistent with Texas Tech University and RCRA (Resource Conservation and Recovery Act) regulations. While handling Boiun's fixative or formalin in the field, latex surgical gloves (or equivalent) and eye protection must be worn, and handling of such fixatives must be done in a well-ventilated area. In the laboratory, any handling of such materials will be done in a fume hood while wearing lab coat, gloves, and eye protection. CRYSTALIZED PRICRIC ACID IS EXPLOSIVE; DO NOT ALLOW BOUIN'S FIXATIVE TO DRY OR BECOME CRYSTALIZED. Always check the screw cap and threads of any jars containing Bouin's fixative to be sure. If such a situation occurs, do not disturb the crystallized materials. Contact the nearest fire station and/or HAZMAT response personel (while in the field), Baywatch Security Personel (if at LHAAP), or TTU Environmental Health and Safety (if at TTU) immediately and explain the situation.

15.5 Endpoint Analysis

Analysis of samples for perchlorate will be according to SOP AC-2-11 "Analysis of Perchlorate by IC".

Plasma thyroid hormone levels will be determined according to SOP MT-2-10 "Radioimmunoassay for Thyroid Hormones".

Extraction and whole-body analysis of thyroid hormones will follow MT-2-08 "Extraction of Thyroid Hormones From Animal Tissues", MT-2-09 "Ion Exchange Purification of Tissue Extracts for Thyroid Hormone Radioimmunoassay", and MT-2-10 "Radioimmunoassay for Thyroid Hormones".

Analysis of tissues for histology will be according to SOP AQ-2-03 "General Histological Processing of Thyroid Follicles in Small Fish".

16. PROPOSED STATISTICAL METHODS

All data will be checked for normality using the Shapiro-Wilk *W* test. Homogeneity of variances will be checked using (Bartlett's or Lavine's test. Comparisons between sites will be accomplished by Analysis of Variance (ANOVA) for multiple mean comparisons. Correlation coefficients will be used to determine if residue levels correlate with biomarkers, reproductive, and/or population data.

17. REPORT CONTENT/RECORDS TO BE MAINTAINED:

Records to be maintained include information entered on Form No. 181 "Aquatic Sampling Form"; Form No. 182 "Fish Dissection Form" or Form No. 027 "Multiple Fish/Amphibian Dissection/Collection Form". Data on these forms will include identity, number, mass, sex and location of animals captured; and identity, amount and location or water or sediment samples collected. Alternatively, this information may be recorded in QA-approved, bound field notebooks. Additional records to be maintained include Form No. 026 "Aquatic Sample Tracking Log"; Form No. Scale calibration log; Form no. 64 b and 64c "Batched Sample tracking log"; any entries in laboratory and field notebooks; and raw data from perchlorate analysis, thyroid hormone analysis and thyroid histology.

Report content will include presentation of data, interpretation, and discussion of the following endpoints:

Perchlorate concentrations in biota collected and water concentrations of perchlorate at sites from which these biota were collected.
Thyroid hormone concentrations in plasma and/or whole bodies of biota collected.

Description and enumeration of alterations of thyroid hormone structure as revealed by histological analysis.
Interpretation of all data, including statistical results
Discussion of the relevance of findings
List of all SOPs used
List of all personnel

18. RECORDS TO BE MAINTAINED / LOCATION:

A final report will be delivered to the Sponsor on or before 15 November 2001. Copies of all data, documentation, records, protocol information, as well as the specimens shall be sent to the Sponsor, or designated delivery point, upon request. All data, the protocol and the final report shall be archived at the testing facility.

19. QUALITY ASSURANCE:

The Quality Assurance Unit will inspect the study at intervals to insure the integrity of the study. Written records will be maintained indicating but not limited to the following: date of inspection, study inspected, phase inspected, person conducting the inspection, findings and problems, recommended and taken action, and any scheduled re-inspections. Any problems likely to effect study integrity shall be brought to the immediate attention of the Study Director. The Quality Assurance Unit will periodically submit written status reports on the study to management and the Study Director.

20. PROTOCOL CHANGES / REVISIONS:

All changes and/or revisions to the protocol, and the reasons therefore, shall be documented, signed and dated by the Study Director and maintained with the protocol and the Quality Assurance Unit.

A FINAL REPORT ENTITLED:
EFFECTS OF THE PERCHLORATE ANION ON EARTHWORMS

STUDY NUMBER:	PAE-01-01
SPONSOR:	Strategic Environmental Research and Development Program (SERDP) 1155 Herndon Parkway, Suite 900 Herndon, Virginia 20170
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, Texas 79409-1163
TEST SITE:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, Texas 79409-1163
ANALYTICAL TEST SITE:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
RESEARCH INITIATION:	1/1/01
RESEARCH COMPLETION:	12/31/01

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List of Tables and Figures

GOOD LABORATORIES PRACTICES STATEMENT

This project, entitled "Effects of the Perchlorate Anion on Earthworms", was performed whenever possible in the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989.

Submitted By:

Todd Anderson
Todd A. Anderson, Ph.D

3-28-02
Date

1.0 DESCRIPTIVE STUDY TITLE:

Effects of the Perchlorate Anion on Earthworms

2.0 STUDY NUMBER:

PAE-01-01

3.0 SPONSOR:

Strategic Environmental Research
and Development Program (SERDP)
1155 Herndon Parkway, Suite 900
Herndon, Virginia 20170

4.0 TESTING FACILITY NAME AND ADDRESS:

The Institute of Environmental and Human Health
Texas Tech University
Box 41163
Lubbock, Texas 79409-1163

5.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:

Start: 1/1/01
Termination: 12/31/01

6.0 KEY PERSONNEL:

Todd Anderson, Analytical Chemist
Ryan Bounds, Quality Assurance Officer
Ron Kendall, Principal Investigator

7.0 STUDY OBJECTIVES / PURPOSE:

To determine the potential effects of the perchlorate anion on earthworm (*Eisenia foetida*) survival and reproduction.

8.0 STUDY SUMMARY:

Potential effects of the perchlorate anion on *Eisenia foetida* were evaluated in two types of tests: a dermal contact test and an artificial soil test. These tests were conducted to determine the likelihood of perchlorate being toxic to earthworms in soil or to affecting reproduction (coccoon production) in earthworms. Both studies contained a range of perchlorate concentrations and multiple replicates per concentration.

Percent survival was examined in 14-day dermal contact tests similar to those described by OECD guidelines (OECD, 1984). In general, perchlorate is relatively non-toxic compared with other types of environmental contaminants. Toxicity was not observed until environmentally relevant concentrations of perchlorate were exceeded by at least an order of magnitude. Overall in the sodium perchlorate filter paper contact test, percent survival decreased as

concentration of perchlorate increased with no earthworms surviving fourteen days in the highest treatment groups (> 700 ppm). In the ammonium perchlorate filter paper contact test, percent survival was nearly consistent for the first four treatment groups. No earthworms survived fourteen days in the higher treatment groups (> 1000 ppm).

The effect of sodium perchlorate on the reproduction of *Eisenia foetida* was also examined. The test involved exposing earthworms to treated soil (van Gestel et al., 1989) that had been dosed with a variety of perchlorate concentrations. Over the four-week test periods, earthworm weights were reduced, on average, in each treatment group. Production of cocoons was observed in four of the five treatment groups with no production in the uppermost treatment group (1000 ppm). Cocoon production was highest in the control group. Production in subsequent treatment groups decreased. As was the case with the dermal contact toxicity tests, perchlorate did not affect earthworm reproduction at environmentally relevant soil concentrations.

9.0 TEST MATERIALS:

Test Material: Soil and earthworms (*Eisenia foetida*)

Test Chemical: Sodium Perchlorate
CAS Number : 7601-89-0
Characterization: NIST Certified.
Source: AccuStandard, Inc.

Test Chemical: Sodium Perchlorate
CAS Number : 7601-89-0
Characterization: ACS Certified.
Source: Fisher Scientific, Inc.

Test Chemical: Ammonium Perchlorate
CAS Number : 7790-98-9
Characterization: ACS Certified.
Source: Aldrich, Inc.

Reference Chemical: deionized water (18M:)
CAS Number : NA
Characterization: The quality of the water was confirmed by analytical tests.
Source: Milli-Q

10.0 JUSTIFICATION OF TEST SYSTEM:

Most forms of perchlorate in the environment exist as the free anion in solution. Soils have very little anion exchange capacity, therefore perchlorate absorbs weakly to most soil minerals, and consequently, is highly mobile in soil and biologically available for uptake into soil-dwelling organisms.

Earthworms play a critical role in topsoil development and conservation. As earthworms burrow, they digest dead and decaying organic matter in the soil. This material is converted into castings. Injection of castings into soil causes buildup of topsoil. Moreover, vegetation grown within soil containing castings enriched in organic matter tends to grow larger and is less susceptible to injury from insects and diseases.

The earthworm species selected was *Eisenia foetida.* Although this is not a common soil species, *Eisenia foetida* occurs in soils rich in organic matter. They are available commercially and breed readily in a variety of organic waste materials. *Eisenia's* susceptibility to chemical contaminants resembles that of true soil-inhabiting species and thus effects on *Eisenia* can be extrapolated to field conditions. Commonly called red wigglers, this species of earthworm is a hardy organism suitable for tests requiring large numbers of subjects and low natural mortality rates (OECD, 1984).

Because they consume mineral particles, microorganisms, and decaying vegetable and animal matter, earthworms are exposed to soil contaminants, such as perchlorate, through ingestion and absorption. While exposure can potentially cause harm to the earthworm, accumulation of perchlorate may occur and possibly have consequences on the numerous other invertebrates and vertebrates that feed on earthworms. These studies are beneficial because data gaps exist on the effects of perchlorate on soil invertebrates as well as the possibility of trophic transfer of perchlorate up the terrestrial food chain (Smith et al., 2001).

11.0 TEST ANIMALS:

NA.

12.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

12.1 Acute Toxicity Test.

Each vial was labeled with an identification name corresponding to the concentration of perchlorate that was used to treat the filter paper within the vial. Identification names correspond to entries on sample log sheets containing additional information including earthworm pre-exposure and post-exposure weights and date of death.

12.2 Reproduction Toxicity Test.

Each jar was labeled with an identification name corresponding to the concentration of perchlorate that was used to treat the artificial soil within the jar. Identification names correspond to entries on sample log sheets containing additional information including earthworm pre-exposure and post-exposure weights and number of cocoons produced.

13.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

13.1 Acute Toxicity Test.

The filter paper contact test (OECD, 1984) involved exposing earthworms to perchlorate on moist filter paper. A range of seven perchlorate concentrations was used in these tests. Five replicates were also constructed per concentration. Perchlorate concentrations were verified by ion chromatography.

13.2 Reproduction Toxicity Test.

The artificial soil test (van Gestel et al., 1989) involved keeping earthworms in artificial soil in which a range of five concentrations of perchlorate was applied. Four replicates were constructed for each treatment group and 10 earthworms were utilized per each replicate.

14.0 METHODS:

14.1 Acute Toxicity Test.

The filter paper contact test involved exposing earthworms to a test compound (perchlroate) on moist filter paper in order to identify potential toxic effects to earthworms in soil (OECD, 1984). Earthworms are capable of absorbing chemicals through respiratory exchange by diffusion through the skin, which is closely underlain by capillary networks. The capillaries allow the circulating blood to attain oxygen and eliminate carbon dioxide through the moist body surface (Gaddie and Douglas, 1975). Both sodium perchlorate and ammonium perchlorate were examined as test substances.

The test species selected was *Eisenia foetida*. Although this is not a common soil species, *Eisenia foetida* occurs in soils rich in organic matter. They are available commercially and breed readily in a variety of organic waste materials. Commonly called red wigglers, this species of earthworm is prolific. Moreover, *Eisenia's* susceptibility to chemicals resembles that of true soil-inhabiting species (OECD, 1984). The earthworms for these tests were purchased from Gib Van Hill, Alvord, IA.

Adult *Eisenia foetida* (those with distinguishable clitella), were exposed to one of seven concentrations of sodium or ammonium perchlorate. For each treatment (consisting of 1 worm per vial), 5 replicates were constructed. More than one worm in a vial was not recommended because the death of a worm might have adverse effects on other worms in the same vial (OECD, 1984). Control vials were treated with MilliQ water without perchlorate. Controls also consisted of 5 replicates. Worms were depurated for 24 hours before being placed in test vials.

The earthworms were exposed to the test substances or MilliQ water in flat-bottom, 60-mL Opticlear glass vials as described by OECD guidelines (OECD, 1984). OECD recommends lining the vials with filter paper and then pipetting 1 mL of the substance into each test vial and evaporating to dryness under a steady stream of filtered compressed air (OECD, 1984).

The above spiking procedure was modified somewhat for our studies. One mL of the test substances was added to flat sheets of filter paper in Petri dishes. The control vials were treated with 1 mL of MilliQ water. The filter papers were allowed to dry under a hood for several hours. The filter papers were then rolled and placed in labeled, 60-mL Opticlear glass vials. Next, 1 mL of MilliQ water was added to each vial to moisten the filter paper. Each vial received 1 pre-weighed earthworm and then was sealed with a screw cap. The vials were placed horizontally in baskets and incubated in the dark in a temperature-controlled cabinet.

Mortality was the clinical endpoint examined. Earthworms were classified as dead when they did not respond to a gentle mechanical stimulus to the anterior end. Deceased worms were removed from the vials, placed in sterile WhirlPak bags, and stored until termination of the experiment.

14.2 Reproduction Toxicity Test.

Reproduction is a sensitive parameter and is of importance for the preservation of populations. Using a modified test procedure developed by van Gestel and co-workers (1989), the effect of sodium perchlorate on reproduction in *Eisenia foetida* was examined. The test involved exposing earthworms to soil treated with different concentrations of perchlorate over 4 weeks and monitoring the production of earthworm cocoons.

14.2.1 Artificial Soil.

The artificial soil was comprised of (by dry weight) 10% sphagnum peat, 20% kaolin clay, and 69% fine sand (van Gestel et al., 1989). The peat moss was strained with a one-millimeter sieve. A total of 10 kg of artificial soil was prepared for the experiment. Cow manure (purchased commercially) was added to each treatment jar at the beginning of the 4 week experiments to serve as a food source.

14.2.2 Earthworms.

The test species selected was *Eisenia foetida*. The earthworms for this test were purchased from Gib Van Hill, Alvord, IA.

14.2.3 Procedure.

Before exposure, earthworms were acclimilated for 1 week in untreated (no perchlorate) artificial soil. Earthworms were then exposed for 3 weeks to the test substance (perchlorate) incorporated in the artificial soil. Typically, tests were preformed at 4 treatment concentrations and a control. The tests included 4 replicates per concentration, with each replicate jar containing 10 earthworms.

As recommended by van Gestel and co-workers (1989), 1-L jars

were used to house the soil and 10 earthworms throughout the duration of the experiment. The authors recommended that equivalent amounts of dry artificial soil (400 g) be placed in the glass jars and then spiked with the test substance. However, we found that it was more efficient to combine the replicates of the respective concentrations of the test substance, spike this composite, and then divide the treated, moist soil as evenly as possible between the 4 jar replicates per concentration.

In the middle of the soil, a hole was made and filled with 8 g (dry weight) of finely ground cow manure (food source). The cow manure was moistened with MilliQ water before use. Ten adult earthworms, with well-developed clitella, were added to each jar. Worms were weighed prior to experiment. The jars were loosely covered with screw lids to prevent moisture loss by evaporation and incubated at 22.9 ρ 0.05 θC in an illuminated climatic chamber (12:12 light:dark).

15.0 RESULTS:

15.1 Acute Toxicity Test.

At the conclusion of each of the studies, the percentage of earthworm survival for each treatment group was calculated and graphed using SigmaPlot 2000. The surviving earthworms were also weighed. These post-exposure weights were compared with pre-exposure weights by calculating group means and standard deviations. Weights were also taken for earthworms that had died during the tests. These weights were averaged and also compared against the pre-expose group mean and standard deviation.

In general, perchlorate is relatively non-toxic compared with other types of environmental contaminants (metals, pesticides, etc.). Toxicity was not observed until environmentally relevant soil concentrations of perchlorate (50 ppb to 50 ppm) were exceeded by approximately an order of magnitude (**Figures 1, 2, 3, 4**). Overall in the sodium perchlorate filter paper contact test, percent survival decreased as concentration of perchlorate increased with no earthworms surviving 14 days in the highest treatment groups (> 700 ppm). In the ammonium perchlorate filter paper contact test, percent survival was nearly consistent for the first 4 treatment groups. No earthworms survived 14 days in the higher treatment groups (> 1000 ppm).

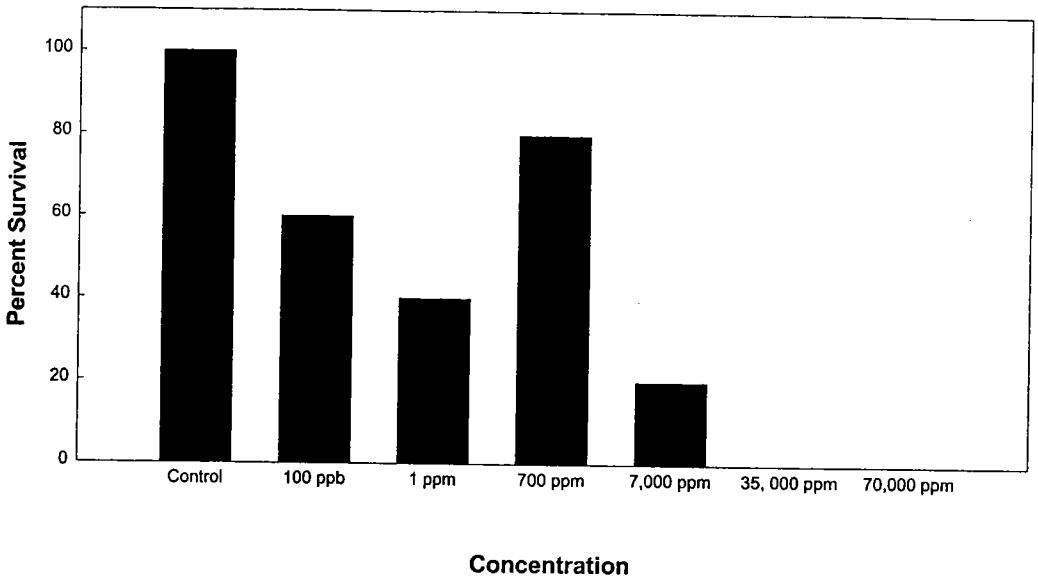

Figure 1. The number of surviving earthworms (*Eisenia foetida*) at a variety of treatment groups following a 14-day dermal contact test using sodium perchlorate. [May 1- 15, 2001]

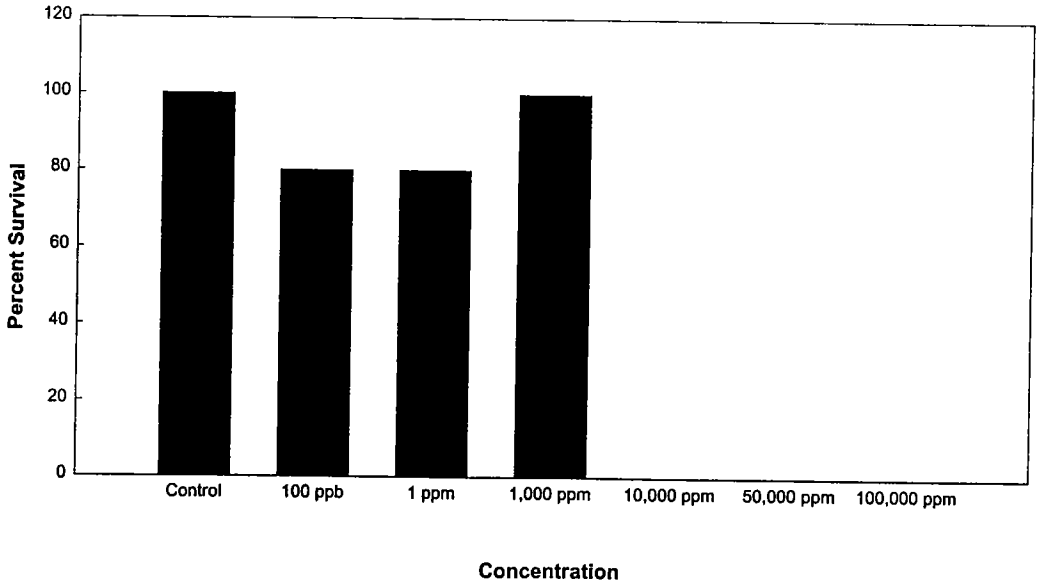

Figure 2. The number of surviving earthworms (*Eisenia foetida*) at a variety of treatment groups following a 14-day filter paper contact test using ammonium perchlorate. [May 22 – June 5, 2001]

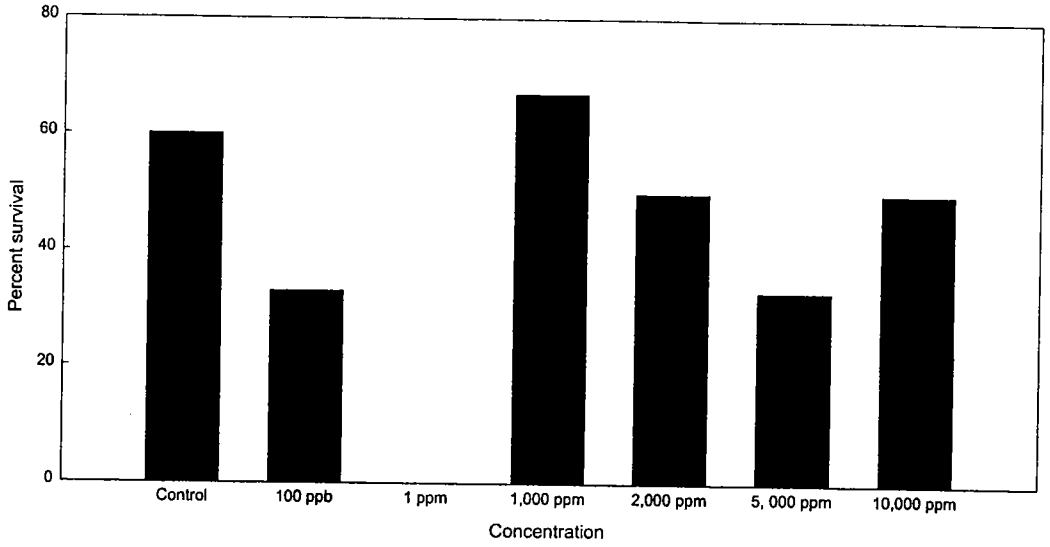

Figure 3. The number of surviving earthworms (*Eisenia foetida*) at a variety of treatment groups following a 14-day filter paper contact test using sodium perchlorate. [October 10 - 24, 2001]

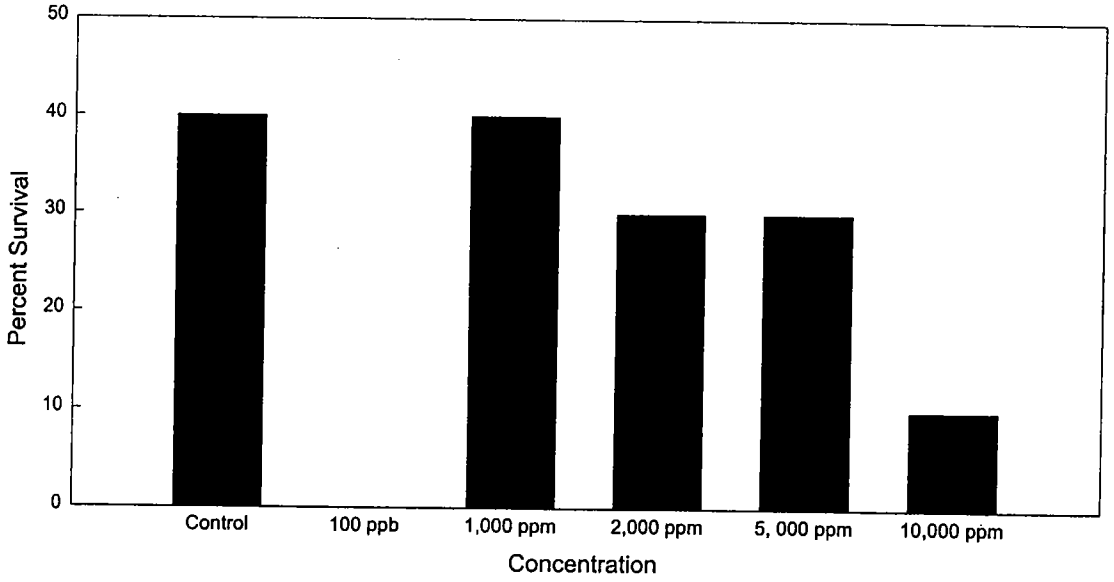

Figure 4. The number of surviving earthworms (*Eisenia foetida*) at a variety of treatment groups following a 14-day filter paper contact test using sodium perchlorate. [November 10 - 24, 2001]

Weights of surviving earthworms also decreased with increasing perchlorate concentrations. However, significant differences were only observed at perchlorate concentrations of little environmental relevance **Figures 5, 6, 7**).

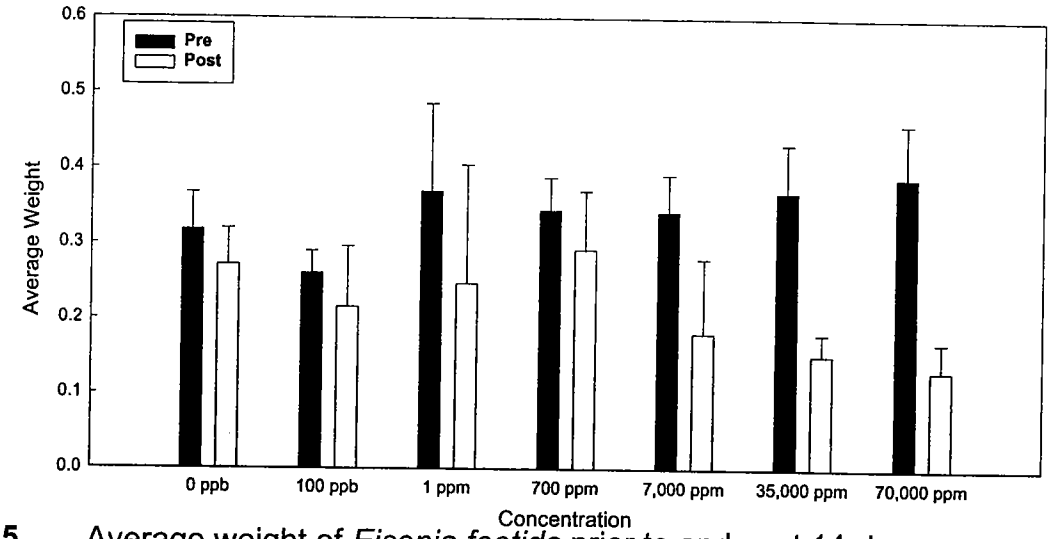

Figure 5. Average weight of *Eisenia foetida* prior to and post 14-day exposure to sodium perchlorate treated filter paper. Post averages include weights from both surviving earthworms and those that died throughout the test. [May 1 – 15, 2001]

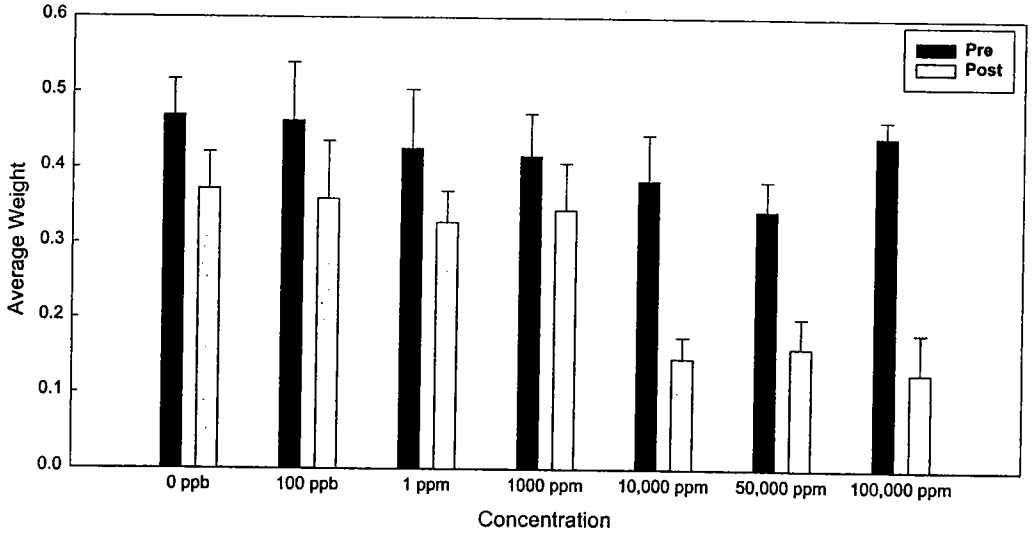

Figure 6. Average weight of *Eisenia foetida* prior to and post 14-day exposure to ammonium perchlorate treated filter paper. Post averages include weights from both surviving earthworms and those that died during the test. [May 22 – June 5, 2001]

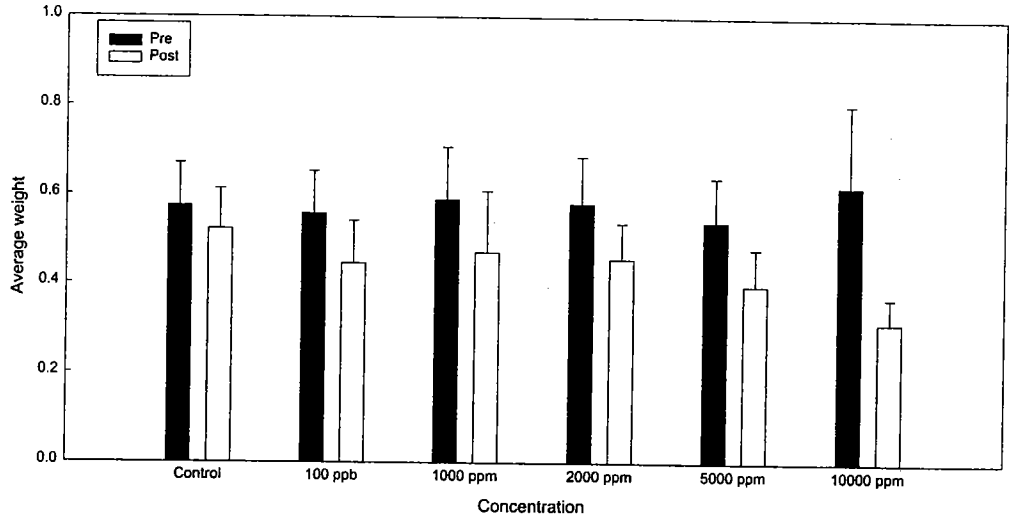

Figure 7. Average weight of *Eisenia foetida* prior to and post 14-day exposure to sodium treated filter paper. Post averages include weights from both surviving earthworms and those that died during the test. [November 10 - 24, 2001]

15.2 Reproduction Toxicity Test.

At the end of the reproductive toxicity testing period (4 weeks), the number of cocoons produced, the number of juvenile worms, the number of alive and dead adult worms, and worm weights were determined.

The number of alive, adult earthworms was first established. Those earthworms were weighed and placed in a separate container. Weights of the earthworms in each treatment group were averaged and compared to the corresponding pre-exposure group mean (**Figure 8**). Although there was a trend towards decreasing post-exposure earthworm weight in a concentration dependent manner, the trend was not statistically significant. These results are consistent with the post-exposure weight results of the dermal toxicity experiments.

In order to determine cocoons and juvenile worms produced, the substrate had to be searched. Two 0.5-mm sieves were placed on top of each other. The artificial soil was washed through these sieves with a stream of water, leaving young worms and cocoons on the sieve, mainly the upper sieve. After the soil substrate was washed through the sieve, juveniles and cocoons were counted. The average number of cocoons produced for each treatment group was calculated and also graphed.

Overall, coccoon production was low during the reproduction experiment,

making the determination of possible perchlorate effects difficult to assess (**Figure 9**). However, there were no cocoons produced in jars containing the highest concentration of perchlorate (1000 ppm).

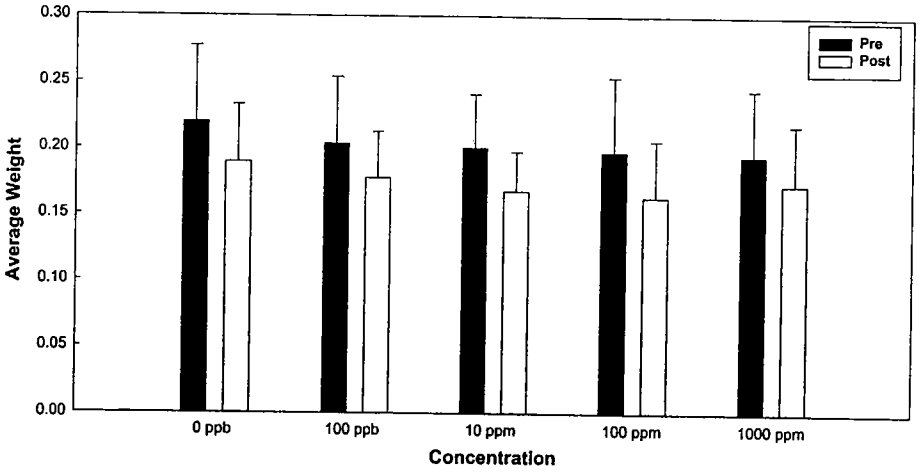

Figure 8. Average weight of *Eisenia foetida* before and after 4-week exposure to perchlorate-treated soil. [August 11 – September 14, 2001]

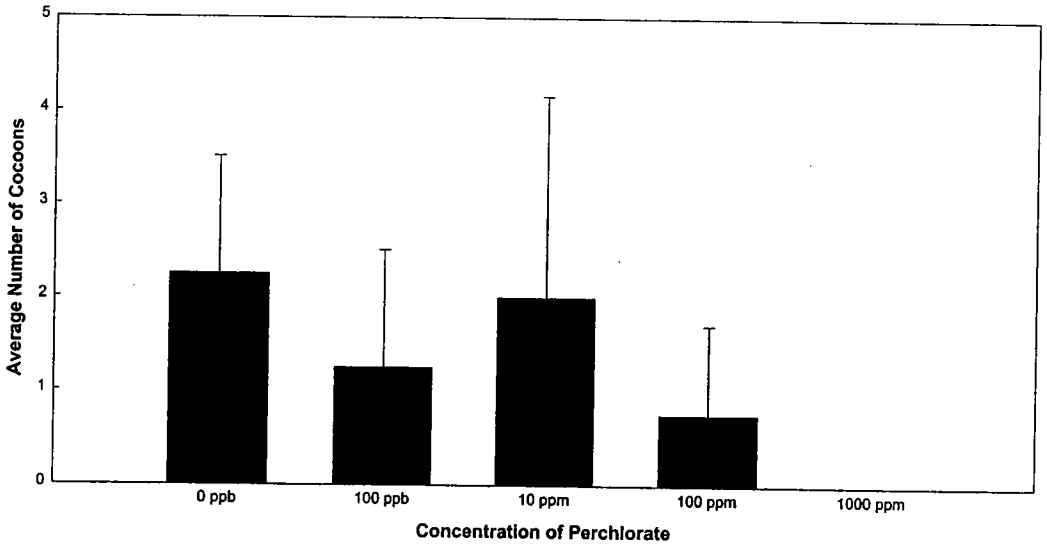

Figure 9. The average number of cocoons produced by colonies of *Eisenia foetida*. These colonies were exposed to various concentrations of perchlorate for 4 weeks. [August 11 – September 14, 2001]

To determine the influence of prior exposure to perchlorate on cocoon hatchability, cocoons produced in each treatment soil were incubated for 5

weeks in untreated (no perchlorate), artificial soil. At the conclusion of the 5-week period, the soil was searched and the number of cocoons and juveniles determined. Although the number of cocoons available for the opportunistic test limited any conclusions from the data (**Figure 10**), we are currently following up these tests by incubating cocoons in contaminated soil and monitoring hatching success.

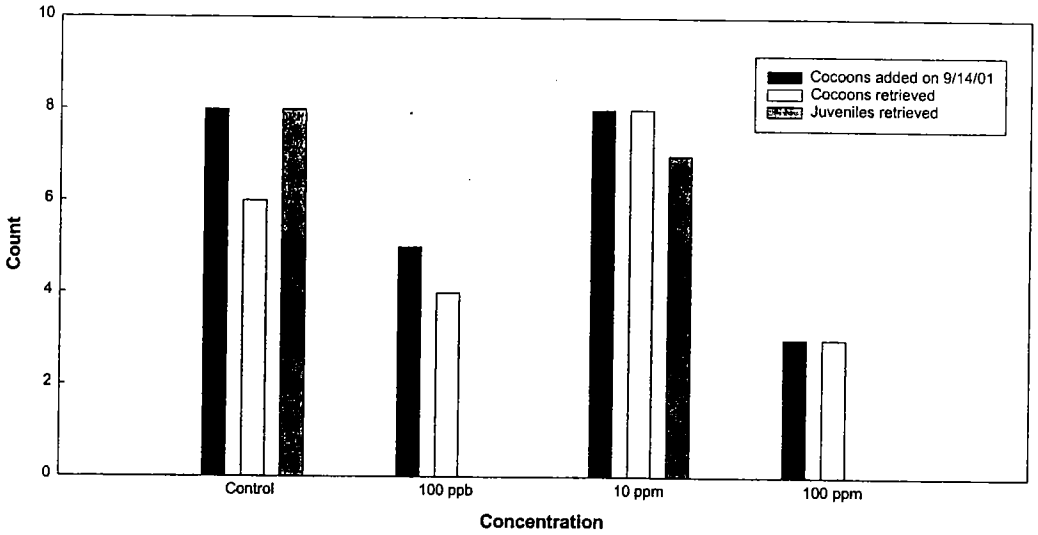

Figure 10. The number of hatched juveniles from cocoons incubated in clean artificial soil for 5 weeks.

Because of some of the problems experienced with worm death in the controls used in the filter paper contact test, and lack of cocoon production by control worms in the artificial soil, we conducted a combination experiment which monitored toxicity and reproduction at various perchlorate concentrations in sand. Results of this study (**Figures 11, 12, 13**) were much more consistent with the concept of a dose-reponse, but were also consistent with the results described earlier.

16.0 DISCUSSION:

16.1 Acute Toxicity Test.

Most forms of perchlorate in the environment exist as the free anion in solution. Soils have very little anion exchange capacity, therefore perchlorate absorbs weakly to most soil minerals, and consequently, is highly mobile and biologically available in soil and groundwater systems (USEPA, 1998).

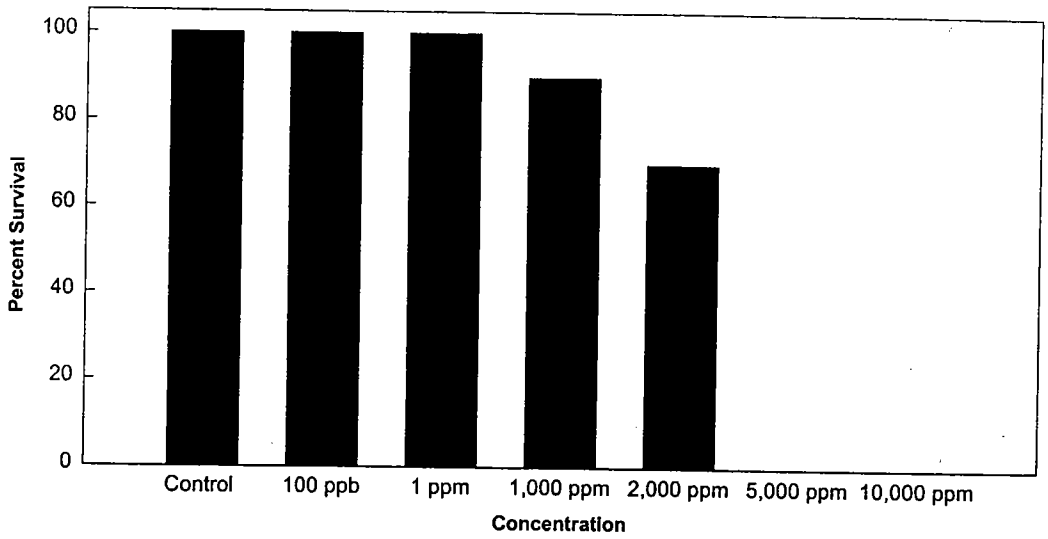

Figure 11. The percentage of surviving earthworms (*Eisenia foetida*) at a variety of treatment groups following a 14-day toxicity test using sodium perchlorate spiked in sand.

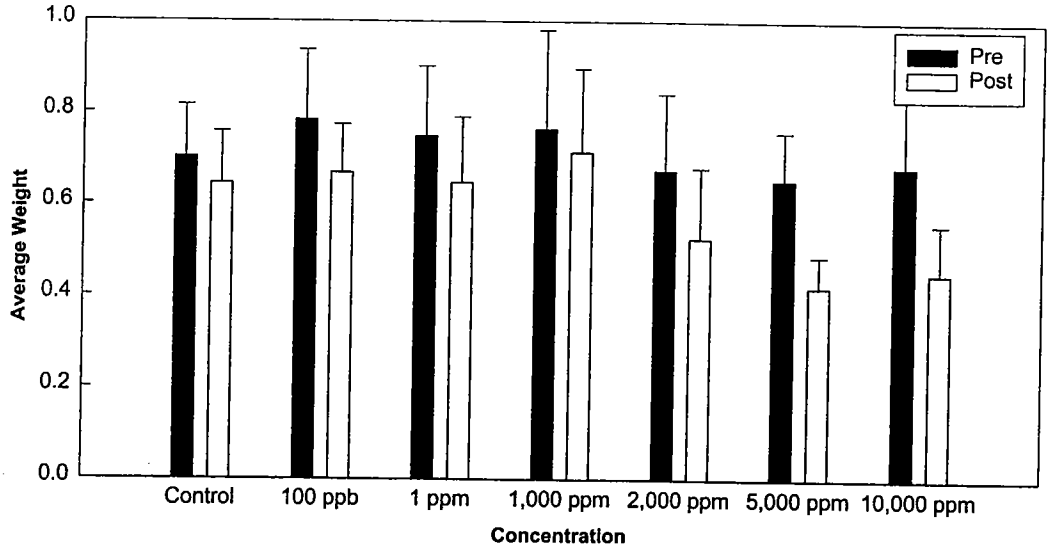

Figure 12. Average weight of *Eisenia foetida* before and after 2-week exposure to perchlorate-treated sand.

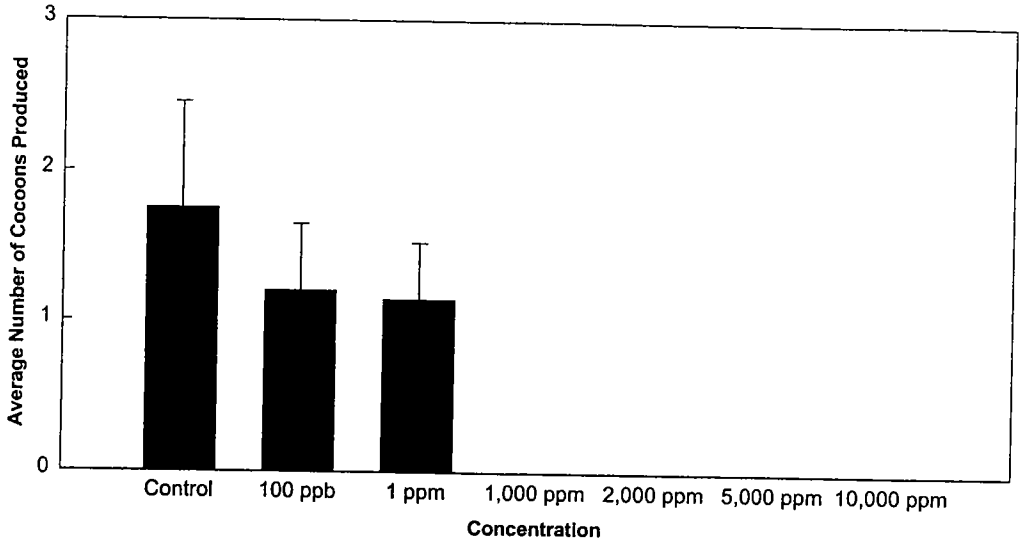

Figure 13. The average number of cocoons produced by colonies of *Eisenia foetida*. These colonies were exposed to various concentrations of perchlorate in sand for 2 weeks.

Earthworms consume mineral particles, microorganisms, and decaying vegetable and animal matter, and are therefore potentially exposed to soil contaminants, such as perchlorate, through ingestion and absorption. Such exposure can be detrimental to earthworm survival and conceivably cause adverse effects to other invertebrates and vertebrates that prey upon earthworms.

Earthworm mortality was examined in 14-day filter paper contact tests with sodium and ammonium perchlorate. Overall in the sodium perchlorate filter paper contact test, percent survival decreased as concentration of perchlorate increased with no earthworms surviving 14 days at the highest treatment groups. This trend is consistent with a dose-response relationship. The noticeable exception was that earthworm survival in the 700-ppm treatment increased slightly, which obscured the general trend.

In the ammonium perchlorate filter paper contact test, percent survival was nearly consistent among the first 4 treatment groups. The control and 1000-ppm treatment groups had 100% survival. Between these treatments, an earthworm in both the 100 ppb and 1 ppm treatment

groups expired, which lowered percent survival to 80%. No earthworms survived 14 days in the higher treatment groups.

Earthworms at the lower perchlorate concentrations consistently survived at percentages equal to the control worms indicating that perchlorate is likely to have little acute toxicity to soil invertebrates at environmentally relevant soil concentrations. In addition, both laboratory and field (Longhorn Army Ammunition Plant) data (not shown) suggest that perchlorate is not likely to accumulate in earthworms to an extent that trophic transfer would be an issue, especially at environmentally relevant perchlorate concentrations.

16.2 Reproduction Toxicity Test.

Using a modified test procedure developed by van Gestel and co-workers (1989), the effect of sodium perchlorate on reproduction in *Eisenia foetida* was examined. Reproduction is a significant factor in the preservation of species. The test involved exposing earthworms to treated soil that had been dosed with different concentrations of sodium perchlorate.

Over the 4-week test period, earthworm weight was reduced, on average, in each treatment group, although this reduction was not statistically significant at concentrations of perchlorate expected to occur in the environment. Production of cocoons was observed in 4 of the 5 treatment groups with no production in the uppermost treatment group (1000 ppm). Cocoon production was highest in the control group. Production in subsequent treatment groups decreased. Unexplainably, the 10 ppm treatment group produced the second highest average number of cocoons. Nonetheless, even small percent decreases in cocoon production could potentially have further reaching effects on population stability, sustainability, and ultimate survival. This is especially true if cocoons from earthworms exposed to perchlorate have lower viabilities than cocoons from earthworms without previous perchlorate exposure.

17.0 STUDY RECORDS AND ARCHIVE:

Study Records will be maintained at The Institute of Environmental and Human Health (TIEHH) Archive for a minimum of one year after study completion date.

18.0 REFERENCES:

Gaddie, R.E., Sr., and D. E. Douglas. 1975. *Earthworms For Ecology and Profit.* Bookworm Publishing Company, Ontario, California.

"OECD Guidelines for Testing of Chemicals, No. 207, Earthworm, Acute Toxicity Test," OECD Publications, Paris, France, April 4, 1984.

Smith, P. N., C. W. Theodorakis, T. A. Anderson, and R. J. Kendall. 2001. Preliminary assessment of perchlorate in ecological receptors at the Longhorn Army Ammunition Plant (LHAAP), Karnack, Texas. *Ecotoxicology.* 10:305-313.

U.S. Environmental Protection Agency (USEPA). 1998. Perchlorate Environmental Contamination: Toxicological Review and Risk Characterization Based on Emerging Information. Office of Research and Development, Washington, DC. NCEA-1-0503.

van Gestel, C. A. M., van Dis, W. A., van Breemen, E. M., and Sparenburg, P. M. (1989). Development of a standardized reproduction toxicity test with the earthworm species *Eisenia foetida andrei* using copper, pentachlorophenol, and 2,4-dichloroaniline. *Ecotoxicology and Environmental Safety* 18:305-312.

Table A1: Average weight of surviving earthworms for $NaClO_4^-$ filter paper contact test

Earthworm	Dose	Initial Weight (g) *050101*	Final Weight (g)	Date of Death
F1	50,000 ppm	0.3578	Died prior to termination of experiment on 051501	
F3	50,000 ppm	0.3780		
F4	50,000 ppm	0.4502		
F5	50,000 ppm	0.3844		
G1	100,000 ppm	0.3507		
G2	100,000 ppm	0.4133		
G3	100,000 ppm	0.4019		
G4	100,000 ppm	0.4829		
G5	100,000 ppm	0.2941		
E3	10,000 ppm	0.2913		
F2	50,000 ppm	0.2733		
C1	1 ppm	0.2741		
E5	10,000 ppm	0.2972		
E1	10,000 ppm	0.3411		
E2	10,000 ppm	0.3912		
D5	1,000 ppm	0.2876		
B4	100 ppb	0.2355		
C3	1 ppm	0.2760		
C5	1 ppm	0.3147		
B5	100 ppb	0.2658		
A1	Control	0.3022	0.2485	Sacrificed 051501
A2	Control	0.3058	0.2388	Sacrificed 051501
A3	Control	0.3981	0.3420	Sacrificed 051501
A4	Control	0.3196	0.3009	Sacrificed 051501
A5	Control	0.2650	0.2307	Sacrificed 051501
B1	100 ppb	0.3048	0.3064	Sacrificed 051501
B2	100 ppb	0.2319	0.2095	Sacrificed 051501
B3	100 ppb	0.2661	0.2772	Sacrificed 051501
C2	1 ppm	0.5312	0.4403	Sacrificed 051501
C4	1 ppm	0.4524	0.3883	Sacrificed 051501
D1	1,000 ppm	0.3919	0.3436	Sacrificed 051501
D2	1,000 ppm	0.3765	0.3550	Sacrificed 051501
D3	1,000 ppm	0.3141	0.2892	Sacrificed 051501
D4	1,000 ppm	0.3533	0.3134	Sacrificed 051501
E4	10,000 ppm	0.3932	0.3530	Sacrificed 051501
Average:		**0.34192**	**0.30912**	
Standard deviation:		**0.071623**	**0.063235**	

Table A2: Average weight of earthworms that died during the $NaClO_4^-$ filter paper contact test

Earthworm	Dose	Initial Weight (g) *050101*	Final Weight (g)	Date of Death
F1	50,000 ppm	0.3578	0.1611	050201
F3	50,000 ppm	0.3780	0.1430	050201
F4	50,000 ppm	0.4502	0.1725	050201
F5	50,000 ppm	0.3844	0.1764	050201
G1	100,000 ppm	0.3507	0.1143	050201
G2	100,000 ppm	0.4133	0.1376	050201
G3	100,000 ppm	0.4019	0.1509	050201
G4	100,000 ppm	0.4829	0.1767	050201
G5	100,000 ppm	0.2941	0.0755	050201
E3	10,000 ppm	0.2913	0.1150	050301
F2	50,000 ppm	0.2733	0.1068	050401
C1	1 ppm	0.2741	0.0899	050501
E5	10,000 ppm	0.2972	0.1214	050501
E1	10,000 ppm	0.3411	0.1342	050601
E2	10,000 ppm	0.3912	0.1817	050901
D5	1,000 ppm	0.2876	0.1615	051101
B4	100 ppb	0.2355	0.1029	051201
C3	1 ppm	0.2760	0.1316	051401
C5	1 ppm	0.3147	0.1863	051401
B5	100 ppb	0.2658	0.1836	051501
A1	Control	0.3022	Survived until termination of experiment on 051501	
A2	Control	0.3058		
A3	Control	0.3981		
A4	Control	0.3196		
A5	Control	0.2650		
B1	100 ppb	0.3048		
B2	100 ppb	0.2319		
B3	100 ppb	0.2661		
C2	1 ppm	0.5312		
C4	1 ppm	0.4524		
D1	1,000 ppm	0.3919		
D2	1,000 ppm	0.3765		
D3	1,000 ppm	0.3141		
D4	1,000 ppm	0.3533		
E4	10,000 ppm	0.3932		
Average:		**0.34192**	**0.141145**	
Standard deviation:		**0.071623**	**0.033569**	

Table A3: Average weight of surviving earthworms for $NH_4ClO_4^-$ filter paper contact test

Earthworm	Dose	Initial Weight (g) *052201*	Final Weight (g)	Date of Death
M1	50,000 ppm	0.3217	Died prior to termination of experiment on 060501	
M2	50,000 ppm	0.3603		
M3	50,000 ppm	0.2956		
M4	50,000 ppm	0.3992		
M5	50,000 ppm	0.3433		
N1	100,000 ppm	0.4602		
N2	100,000 ppm	0.4568		
N3	100,000 ppm	0.4125		
N4	100,000 ppm	0.4287		
N5	100,000 ppm	0.4574		
L3	10,000 ppm	0.3114		
L5	10,000 ppm	0.4457		
L2	10,000 ppm	0.4487		
L1	10,000 ppm	0.3591		
I4	100 ppb	0.4464		
J2	1 ppm	0.5132		
L4	10,000 ppm	0.3537		
H1	Control	0.4550	0.3900	Sacrificed 060501
H2	Control	0.3927	0.2906	Sacrificed 060501
H3	Control	0.4894	0.3711	Sacrificed 060501
H4	Control	0.4989	0.4199	Sacrificed 060501
H5	Control	0.5116	0.3889	Sacrificed 060501
I1	100 ppb	0.4286	0.3403	Sacrificed 060501
I2	100 ppb	0.5435	0.4403	Sacrificed 060501
I3	100 ppb	0.3589	0.3123	Sacrificed 060501
I5	100 ppb	0.5344	0.4352	Sacrificed 060501
J1	1 ppm	0.4795	0.3632	Sacrificed 060501
J3	1 ppm	0.4496	0.3814	Sacrificed 060501
J4	1 ppm	0.3424	0.2919	Sacrificed 060501
J5	1 ppm	0.3453	0.2959	Sacrificed 060501
K1	1,000 ppm	0.4693	0.3988	Sacrificed 060501
K2	1,000 ppm	0.4783	0.4127	Sacrificed 060501
K3	1,000 ppm	0.3838	0.2866	Sacrificed 060501
K4	1,000 ppm	0.4034	0.3482	Sacrificed 060501
K5	1,000 ppm	0.3477	0.2785	Sacrificed 060501
Average:		**0.4207486**	**0.3581**	
Standard deviation:		**0.0669669**	**0.05457**	

Table A4: Average weight of earthworms that died during the NH_4ClO_4 filter paper contact test

Earthworm	Dose	Initial Weight (g) *052201*	Final Weight (g)	Date of Death
M1	50,000 ppm	0.3217	0.1489	052301
M2	50,000 ppm	0.3603	0.1616	052301
M3	50,000 ppm	0.2956	0.1076	052301
M4	50,000 ppm	0.3992	0.2193	052301
M5	50,000 ppm	0.3433	0.1670	052301
N1	100,000 ppm	0.4602	0.1246	052301
N2	100,000 ppm	0.4568	0.0655	052301
N3	100,000 ppm	0.4125	0.2034	052301
N4	100,000 ppm	0.4287	0.0926	052301
N5	100,000 ppm	0.4574	0.1521	052301
L3	10,000 ppm	0.3114	0.1142	052901
L5	10,000 ppm	0.4457	0.1809	052901
L2	10,000 ppm	0.4487	0.1719	053001
L1	10,000 ppm	0.3591	0.1281	060101
I4	100 ppb	0.4464	0.2669	060201
J2	1 ppm	0.5132	0.3086	060201
L4	10,000 ppm	0.3537	0.1383	060201
H1	Control	0.4550	Survived until termination of experiment on 060501	
H2	Control	0.3927		
H3	Control	0.4894		
H4	Control	0.4989		
H5	Control	0.5116		
I1	100 ppb	0.4286		
I2	100 ppb	0.5435		
I3	100 ppb	0.3589		
I5	100 ppb	0.5344		
J1	1 ppm	0.4795		
J3	1 ppm	0.4496		
J4	1 ppm	0.3424		
J5	1 ppm	0.3453		
K1	1,000 ppm	0.4693		
K2	1,000 ppm	0.4783		
K3	1,000 ppm	0.3838		
K4	1,000 ppm	0.4034		
K5	1,000 ppm	0.3477		
Average:		**0.4207486**	**0.16185**	
Standard deviation:		**0.0669669**	**0.06161**	

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Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: ____ Amendment ____Deviation X Addendums

Document Reference Information
Check One: X Protocol ____ SOP ____ Other _____
Title: Uptake of Ammonium Perchlorate and Thyroid Status in Native Fish
Dated: February 13, 2001
Document # (if appropriate): FISH 01-1
Page #(s): 8
Section #: 15.5
Text to reference: _____

Change in Document: Additional tissue collected in catfish for tissue distribution of perchlorate study: Kidney (KY) and Gonad (GD).

Justification and Impact on Study: It was necessary to collected two additional organs from the catfish to get more information about perchlorate uptake.

Submitted by: Signature: Callie Bradford Date: 9/20/a

Authorized by: Study Director: [signature] Date: 9/27/01

Received by: Quality Assurance Unit: Brian Birdwell Date: 3/28/02

* Sequentially numbered in order of the date that the change is effective

A FINAL REPORT ENTITLED:
UPTAKE OF THE PERCHLORATE ANION INTO VARIOUS PLANT SPECIES

STUDY NUMBER:	PAP-01-01
SPONSOR:	Strategic Environmental and Research Development Program (SERDP) 1155 Herndon Parkway, Suite 900 Herndon, Virginia 20170
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
TEST SITE:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
ANALYTICAL TEST SITE:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
RESEARCH INITIATION:	1/1/2001
RESEARCH COMPLETION:	12/31/2001

Table of Contents

List of Tables and Figures

GOOD LABORATORIES PRACTICES STATEMENT

This project, entitled "Uptake of the Perchlorate Anion into Various Plant Species", was performed whenever possible in the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989

Submitted By:

Todd Anderson
Todd A. Anderson, Ph.D

3-28-02
Date

1.0 DESCRIPTIVE STUDY TITLE:

Uptake of the perchlorate anion into various plant species.

2.0 STUDY NUMBER: PAP-01-01

3.0 SPONSOR:

Strategic Environmental Research and Development Program (SERDP)
1155 Herndon Parkway, Suite 900
Herndon, Virginia 20170

4.0 TESTING FACILITY NAME AND ADDRESS:

The Institute of Environmental and Human Health
Texas Tech University
Box 41163
Lubbock, Texas 79409-1163

5.0 PROPOSED EXPERIMENTAL START & TERMINATION DATES:

Start: 1/1/2001
Termination: 12/31/2001

6.0 KEY PERSONNEL:

Todd Anderson, Analytical Chemist
Ryan Bounds, Quality Assurance Officer
Ron Kendall, Principal Investigator

7.0 STUDY OBJECTIVES / PURPOSE:

To determine the uptake of the perchlorate anion into various types of vegetation.

8.0 STUDY SUMMARY:

Four plant species (cucumber, duckweed, lettuce, and soybean) were used to evaluate perchlorate uptake into plants from surrounding media under laboratory conditions. The media included sand as well as hydroponic systems. Duration of tests varied between plant species and between tests within a species, but typically were conducted over 4 weeks. Plants were extracted using accelerated solvent extraction and analyzed using ion chromatography. In addition to evaluating perchlorate uptake, germination rates of cucumber and lettuce seeds in the presence of perchlorate were also determined.

Uptake and accumulation of perchlorate from sand in vegetation occurred in all plant species. This was shown by perchlorate levels in plant as well as removal of perchlorate from the sand of planted systems. Perchlorate was translocated from roots to leaves and reached a steady state concentration that essentially depleted perchlorate from the sand.

Germination of cucumber seeds was not effected by the presence of perchlorate. However, germination of lettuce seeds was effected.

9.0 TEST MATERIALS:

Test Material: Plants (cucumber, *Cucumis sativus* L., duckweed, *Lemna minor*, lettuce, *Lactuca sativa* L., and soybean, *Glycine max.*

Test Chemical: Sodium Perchlorate
CAS Number : 7601-89-0
Characterization: NIST Certified.
Source: AccuStandard, Inc.

Test Chemical: Ammonium Perchlorate
CAS Number : 7790-98-9
Characterization: NIST Certified.
Source: AccuStandard, Inc.

Reference Chemical: deionized water (18M:)
CAS Number : NA
Characterization: The quality of the water was confirmed by analytical tests.
Source: Milli-Q

10.0 JUSTIFICATION OF TEST SYSTEM:

Perchlorate is highly mobile in soil and groundwater systems, which presents the potential for plants to be exposed to and take up perchlorate. Perchlorate in plants can then lead to trophic transfer to organisms that consume the contaminated plants. Currently, there is little data on uptake of perchlorate into plants. Therefore, data are needed related to the quantitative aspects of perchlorate uptake into vegetation and some of the environmental conditions which might facilitate perchlorate uptake. The plant species used in this study represent some of the standard species used in germination and uptake experiments.

11.0 TEST ANIMALS:

NA.

12.0 PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

Plants were labeled with an identification name corresponding to the type of plant and the concentration at which the test was conducted. Plants that were sampled at certain testing times were labeled consecutively according to type of plant and the testing period. ID names correspond to entries on the sample log sheet containing additional information including weights of the plants and/or specific tissue.

13.0 EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Various types of vegetation were used to evaluate uptake of perchlorate into plants from either water or sand. Hydroponic and sand systems were used to minimize external factors that affect uptake of contaminants into vegetation.

Plants were placed in treatments at varying concentrations of sodium perchlorate or ammonium perchlorate in order to evaluate possible concentration effects. Test lengths varied among plants to monitor the possibility of plant metabolism. Once plants were removed from testing conditions, plants were extracted and analyzed using ion chromatography (IC).

14.0 INTRODUCTION:

Perchlorate originates as a contaminant in the environment from solid salts of ammonium, potassium, and sodium perchlorate. Perchlorate is quite soluble in water and rather resistant to reactions with other constituents in water. For this reason, perchlorate can persist for many decades under typical groundwater and surface water conditions. Perchlorate's water solubility plays a role in the uptake of perchlorate into plants. Recent studies have demonstrated that certain plant species are capable of taking up perchlorate. Susarla et al. (2000) evaluated six vascular plants: sweet gum, black willow, pickleweed, smartweed, fragrant white water-lily, and duckmeat. They found that all plants with the exception of duckmeat were capable of rapid uptake of perchlorate. Another study reported uptake of perchlorate by salt cedars in the Las Vegas Wash (Urbansky et al., 2000).

The focus of this study was to monitor uptake of sodium and ammonium perchlorate into a variety of plant species. This information is important because of the possibility of trophic transfer of perchlorate from plants to animals, including humans. Additionally, there is little data on perchlorate uptake in plants. Four types of plants were used in this study: cucumbers (*Cucumis sativus* L.), duckweed (*Lemna minor*), lettuce (*Lactuca sativa* L.), and soybean (*Glycine max*). Sand and hydroponic systems were used to minimize external factors that affect uptake of contaminants into vegetation. However, we also conducted some preliminary work on the influence of external nutrient levels on perchlorate uptake using a commercial fertilizer (Hyrdosol).

15. 0 METHODS:

15.1 Experimental Setup.

15.1.1 Germination of Plants in the Presence of Perchlorate

A. Cucumber

A seed germination experiment was performed with National Pickling Cucumber seeds to test whether various treatments of sodium perchlorate could effect germination. The seeds were germinated in paper cups containing 50 g of Ottawa sand (Fisher Scientific). It was determined from a preliminary study that it would take 5 mL of liquid to wet 50 g of sand and therefore each cup received 5 mL of the appropriate perchlorate concentration (controls received Milli-Q water only) in Milli-Q water. The control group contained 20 replicates and experimental groups had 6 replicates at perchlorate concentrations of 10, 100, 1000,

and 10,000 ppb. Test conditions were as follows: light intensity = 243 LUX, 15:9 light:dark photoperiod, incubator temperature = 22.8 ρ 0.08 θC.

To determine whether watering was needed each day, 3 cups were randomly selected daily and weighed. The weights of the three cups were averaged and if this value was 1 g or more below the initial weight, all the cups were adjusted to their initial moisture value.

B. Lettuce

A 10 day experiment (similar to the one described above) was conducted to determine lettuce germination rates at different concentrations of sodium perchlorate. Lettuce seeds were placed in polystyrene cups containing 50 g of Ottawa sand (Fisher Scientific) and incubated (22 θC, 15:9 light:dark photoperiod). The concentrations of sodium perchlorate in sand were 0, 10, 100, 1000, and 10,000 ppb. There were 10 replicates in the control (0 ppb) group and 6 replicates in the other groups. The plants were observed twice daily and watered as appropriate to maintain optimal moisture for seed germination.

C. Duckweed

A perchlorate toxicity test was also conducted with duckweed plants, although this test was not a germination test per se. Duckweed plants were exposed to varying concentrations of sodium perchlorate (0, 10, 25, 50, and 100 ppb) in Hydrosol over 5 days. There were three replicates at each concentration. Plant health (coloration) and growth were monitored daily.

15.1.2 Uptake of Perchlorate into Plants

A. Cucumber

A 4 week study was conducted to determine uptake of sodium perchlorate into cucumber and the possible influence of external nutrient concentrations on perchlorate uptake. Cucumber seeds were placed in polystyrene cups containing 100 g of Ottawa sand (Fisher Scientific) and grown in an incubator at 22 θC with a 15:9 light:dark photoperiod. The concentration of sodium perchlorate in the sand was 100 ppb. Controls consisted of 4 cups spiked with perchlorate but left unplanted. Perchlorate concentration in the sand was measured at week 0. In addition, cups with plants and 1 cup without a plant were removed at weeks 1, 2, 3, and 4 for perchlorate analysis. Plants were removed and cut into two

parts: portion of plant above sand level (stem and leaves) and portion of plant below sand level (roots). Each portion of the plant sample was weighed, rinsed with water, and allowed to dry prior to extraction (described below).

The water volume in each cup was determined by weighing the cup and its contents, and then subtracting the original weight of the sand and cup. Then 10 mL of Milli-Q water was added to the sand and mechanically agitated for 1 hour. Water above sand level was sampled (0.5 mL), diluted to 5 mL with Milli-Q water in 5-mL IC vials (Dionex Corporation), and analyzed by IC.

A 2 week study was conducted to determine uptake of sodium perchlorate into cucumbers grown in sand in the presence of DI water. Cucumber seeds were placed in polystyrene cups containing 50 g of Ottawa sand (Fisher Scientific) and grown in an incubator at 22 θC (15:9 light:dark photoperiod). The concentration of sodium perchlorate in the sand was 100 ppb. Controls consisted of 4 cups spiked with perchlorate but left unplanted. Perchlorate concentration in the sand was measured at week 0. Three cups with plants and 1 cup without a plant were removed at weeks 1 and 2. Plants were removed and cut into two parts: portion of plant above sand level (stem and leaves) and portion of plant below sand level (roots). Each portion of the plant sample was weighed, rinsed with water, and allowed to dry prior to extraction (described below).

The water volume in each cup was determined by weighing the cup and its contents and then subtracting the original weight of the sand and cup. Then 10 mL of Milli-Q water was added to the sand and mechanically agitated for 1 hour. Water above sand level was sampled (0.5 mL), diluted to 5 mL with Milli-Q water in 5-mL IC vials (Dionex Corporation), and analyzed by IC.

A 4 week study was conducted to determine uptake of sodium perchlorate into cucumbers grown in sand in the presence of Hydrosol:Milli-Q water (50:50) and Hydrosol:Milli-Q water (25:75). Cucumber seeds were placed in polystyrene cups containing 100 g of Ottawa sand (Fisher Scientific) and grown in an incubator at 22 θC (15:9 light:dark photoperiod. The concentration of sodium perchlorate in the sand was 100 ppb. Controls consisted of 5 cups spiked with perchlorate but left unplanted.

Perchlorate concentration in the sand was measured at week 0 and week 0.5. Four cups with plants and 1 cup without a plant were removed at weeks 1, 1.5, 2, 3, and 4. Plants were removed and cut into two parts: portion of plant above sand level (stem and leaves) and portion of plant below sand level (roots). Each portion of the plant sample was weighed, rinsed with water, and allowed to dry prior to extraction (described below).

The water volume in each cup was determined by weighing the cup and its contents, and then subtracting the original weight of the sand and cup. Then 20 mL of Milli-Q water was added to the sand and mechanically agitated for 1 hour. Water above sand level was sampled (0.5 mL), diluted to 5 mL with Milli-Q water in 5-mL IC vials (Dionex Corporation), and analyzed by IC.

An 8 week study was conducted to determine the time course of uptake of sodium perchlorate into cucumber grown in sand in the presence of Hydrosol. Cucumber seeds were placed in polystyrene cups containing 100 g of Ottawa sand (Fisher Scientific) and grown in an incubator at 22 ϴC (15:9 light:dark photoperiod). The concentration of sodium perchlorate in the sand was 100 ppb. Controls consisted of 9 cups spiked with perchlorate but left unplanted. Perchlorate concentration in the sand was measured at week 0 and week 0.5. Four cups with plants and 1 cup without a plant were removed at weeks 1, 1.5, 2, 3, and 4. The remaining cups were respiked with perchlorate (100 ppb based on sand weight) to determine additional uptake into vegetation. Four cups with plants and one cup without a plant were removed at weeks 5, 6, 7 and 8. Plants were removed and cut into two parts: portion of plant above sand level (stem and leaves) and portion of plant below sand level (roots). Each portion of the plant sample was weighed, rinsed with water, and allowed to dry prior to extraction (described below).

The water volume in each cup was determined by weighing the cup and its contents, and then subtracting the original weight of the sand and cup. Then 20 mL of Milli-Q water was added to the sand and mechanically agitated for 1 hour. Water above sand level was sampled (0.5 mL), diluted to 5 mL with Milli-Q water in 5-mL IC vials (Dionex Corporation), and analyzed by IC.

B. Duckweed

Possible uptake into aquatic vegetation was evaluated initially in a 3 day study with duckweed grown in a Hydrosol solution. There were three replicates at a perchlorate concentration of 100 ppb. On the third day, the plants were removed, weighed, rinsed with water, and allowed to dry prior to extraction.

A time course of perchlorate uptake into duckweed was conducted over a 10 day period. Eight replicates of duckweed plants were set up in Hydrosol at a perchlorate concentration of 100 ppb. Two replicates were removed on days 1, 3, 5, and 10. The plants were weighed, rinsed with water, and allowed to dry prior to extraction.

A 7 day uptake study of sodium perchlorate in DI water was also conducted to determine the possible influence of plant growth in the nutrient solution (Hydrosol). Four replicates were set up at a perchlorate concentration of 100 ppb. Plants were removed, weighed, rinsed with water, and allowed to dry prior to extraction.

Finally, a perchlorate uptake test using duckweed grown in a half-strength Hydrosol solution was conducted over 14 days. There were 4 replicates at a concentration of 100 ppb. Plants were removed, weighed, rinsed with water, and allowed to dry prior to extraction.

C. Lettuce

A 6 week study was conducted to determine uptake of sodium perchlorate into lettuce grown in sand in the presence of Hydrosol. Lettuce seeds were placed in polystyrene cups containing 50 g of Ottawa sand (Fisher Scientific) and grown in an incubator at 22 ϴC (15:9 light:dark photoperiod. The concentration of sodium perchlorate in the sand was 100 ppb. Controls consisted of 4 cups spiked with perchlorate but left unplanted. Perchlorate concentration in the sand was measured at week 0. Five cups with plants and one cup without a plant were removed at weeks 1, 2, 3, 4, 5, and 6. Plants were removed and cut into two parts: portion of plant above sand level (stem and leaves) and portion of plant below sand level (roots). Each portion of the plant sample was weighed, rinsed with water, and allowed to dry prior to extraction (described below).

The water volume in each cup was determined by weighing

the cup and its contents, and then subtracting the original weight of the sand and cup. Then 10 mL of Milli-Q water was added to the sand and mechanically agitated for 1 hour. Water above sand level was sampled (0.5 mL), diluted to 5 mL with Milli-Q water in 5-mL IC vials (Dionex Corporation), and analyzed by IC.

D. Soybean

A 3 week sodium perchlorate uptake test was conducted with soybeans in a recirculating hydroponic system. Soybean seeds were placed in Rockwool blocks and germinated prior to exposure to perchlorate. Germinated plants in Rockwool blocks were placed in gutters at varying concentrations of sodium perchlorate: 0, 25, and 100 ppb. There were 4 control plants and 8 plants for each treatment. The gutters were covered with black-on-white vinyl. During this experiment, blocks were rotated from the top shelf of the incubator to the bottom shelf of the incubator to ensure equal growth of all plants. Plants were incubated at 23.8 ϴC (12:12 light:dark photoperiod). At the conclusion of the experiment, each portion of the plant sample was weighed, rinsed with water, and allowed to dry prior to extraction (described below).

A 4 week study was conducted to determine uptake of sodium perchlorate into soybeans grown in sand in the presence of Hydrosol. Soybean seeds were placed in polystyrene cups containing 50 g of Ottawa sand (Fisher Scientific) and grown in an incubator at 22 ϴC (15:9 light:dark photoperiod). The concentration of sodium perchlorate in the sand was 100 ppb. Perchlorate concentration in the sand was measured at week 0. Eight cups were removed at weeks 1, 2, 3, and 4. Plants were removed and cut into two parts: portion of plant above sand level (stem and leaves) and portion of plant below sand level (roots). Each portion of the plant sample was weighed, rinsed with Milli-Q water, and allowed to dry prior to extraction (described below).

The water volume in each cup was determined by weighing the cup and its contents, and subtracting the original weight of the sand and cup. Then 10 mL of Milli-Q water was added to the sand and mechanically agitated for 1 hour. Water above sand level was sampled (0.5 mL), diluted to 5 mL with Milli-Q water in 5-mL IC vials (Dionex Corporation), and analyzed by IC.

15. 2 Extraction.

All plants were extracted in 11-mL cells with Milli-Q water using a Dionex Accelerated Solvent Extractor (ASE 200) as generally described in **SOP AC-4-04-01**. Cells were filled with Milli-Q water, heated for 5 minutes at 100°C and pressurized at 1500 psi. Extracts were collected in 60 mL collection vials. Extract volume was recorded.

15.3 Clean-up.

For all plants, 1.0 mL of the water extract was cleaned using alumina solid phase extraction (SPE) cartridges. Cleaned extracts were diluted to 5 mL with Milli-Q water and filtered (0.45 Πm) prior to analysis by IC.

15.4 Analysis.

General operation of the ion chromatograph (DX-500, Dionex Corp.) is described in **SOP AC-4-03-01**. The operation of the ion chromatograph for perchlorate analysis is described in **SOP AC-2-11-01**. These SOPs provided the basis for determining perchlorate in water and plant extracts. As described in **SOP AC-2-11-01**, the analysis of perchlorate using the Dionex instrument is controlled by PeakNet software using a method within the software package entitled "Perchlorate 1a". The methods, along with the analytical data quality tests, have been detailed in previous reports.

15.5 Field Collection Analysis.

Plant and soil samples were collected from Longhorn Army Ammunition Plant (LHAAP) in order to assess perchlorate uptake in the field. Samples were collected from around Building 25C (former perchlorate grinding facility) and the INF pond. Plants were extracted and analyzed as described above for the laboratory studies. For soil samples, 12 g of soil was placed in 4 oz. glass jars and 10-15 mL Milli-Q water was added to the soil. jars were mechanically agitated for 30 minutes. Extract (0.5 mL) was removed, diluted to 5.0 mL with Mill-Q water, filtered (0.45 Πm), and analyzed by IC.

16.0 RESULTS AND DISCUSSION:

16.1 Toxicity

16.1.1 Cucumber

Sodium perchlorate had no effect on germination of cucumber seeds at the concentrations tested. Statistically, there was no difference in percent germination among the treatments (**Figure 1**).

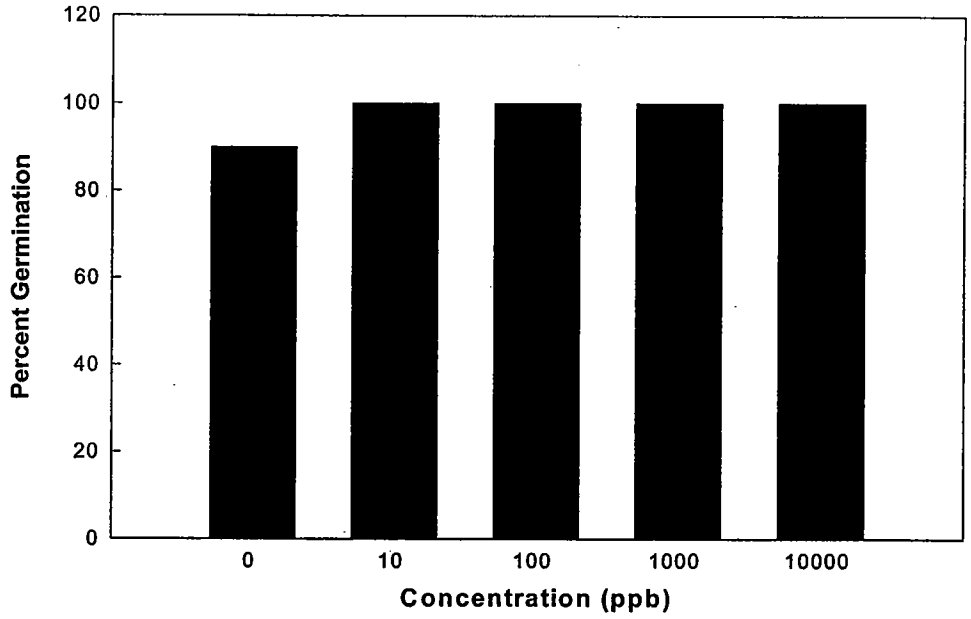

Figure 1. Percent germination of cucumber seeds (*Cucumis sativus* L.) following a 10 day incubation period in sand treated with sodium perchlorate. There were 20 replicates in the control group and 6 replicates in each of the 4 treatment groups.

16.1.2 Lettuce

The 10,000 ppb (10 ppm) perchlorate treatment had a significant effect on lettuce germination. There was a 60% decrease of germination rate in the 10 ppm treatment compared to the other 4 treatment groups (**Table 1**). However, this concentration has little environmental relevance.

Table 1. Germination rates for lettuce (*Lactuca sativa* L.) in sand treated with sodium perchlorate. The duration of this experiment was 10 days.

Perchlorate treatment	# of samples	# germinated	Percent germination
0 ppb	10	8	80.0
10 ppb	6	5	83.3
100 ppb	6	5	83.3
1000 ppb	6	6	83.3
10000 ppb	6	2	33.3

16.1.3 Duckweed

For the toxicity test on duckweed, ANOVA was used to determine if there was any effect of the perchlorate concentrations on growth of the plant. Results of the ANOVA indicated that there was no significant difference in biomass between treatment groups after 5 days (**Figure 2**) despite some depression in biomass with increasing perchlorate concentration.

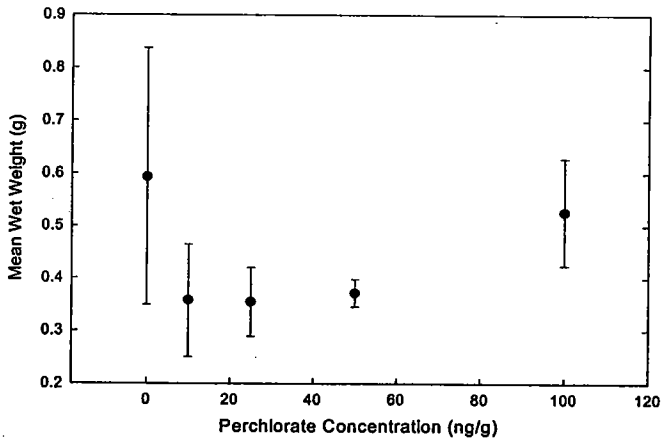

Figure 2. Relationship between nominal concentrations of sodium perchlorate in the toxicity test and the average wet weight of duckweed plants after a 5 day incubation period. Error bars represent one standard deviation of the mean.

16.2 Uptake Data

16.2.1 Cucumber

From our unplanted sand data in the 4 week perchlorate uptake study in the presence of Hydrosol, there was some degradation of perchlorate over time (from 111 ppb to 99 ppb over 4 weeks). There was also considerable uptake of perchlorate into cucumber leaves and stems (**Table 2, Figure 4**). Perchlorate concentrations

in planted sand were not detected (ND) by week 3 (**Figure 3**).

Table 2. Uptake of sodium perchlorate from sand into cucumber (*Cucumis sativus* L.) in the presence of Hydrosol. Plant concentrations were calculated based on dry weight. Depending on the success of the seed germination, the number of replicates varied at each sampling point.

	Unplanted Sand Conc (ppb)	Planted Sand Conc (ppb)	Root Conc (ppb)	Leaf Conc (ppb)
Week 0	111 ± 5	114 ± 2	ND	ND
Week 1	NS	115 ± 14	221162 ± 38915	34963 ± 9052
Week 2	108 ± 0.68	9	150763	20665
Week 3	NS	ND	ND	22083 ± 17135
Week 4	99	ND	ND	41060 ± 32011

ND = not detected; NS = sample was not collected at this testing period

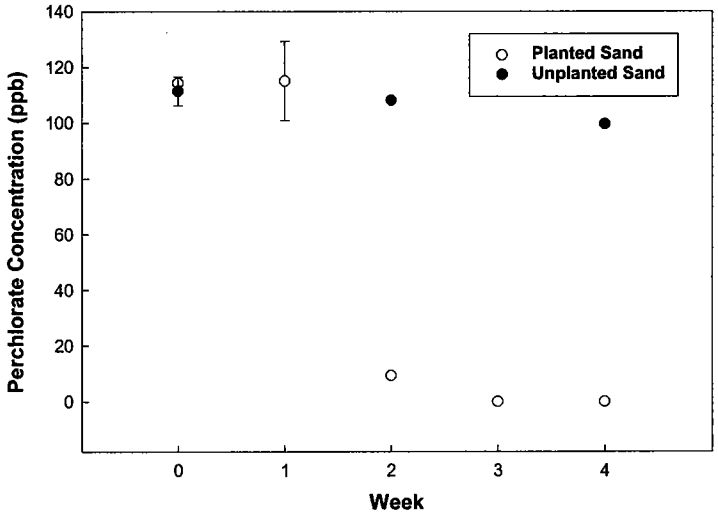

Figure 3. Perchlorate concentrations in sand from 4 week cucumber (*Cucumis sativus* L.) perchlorate uptake experiment in the presence of Hydrosol. Error bars represent one standard deviation of the mean.

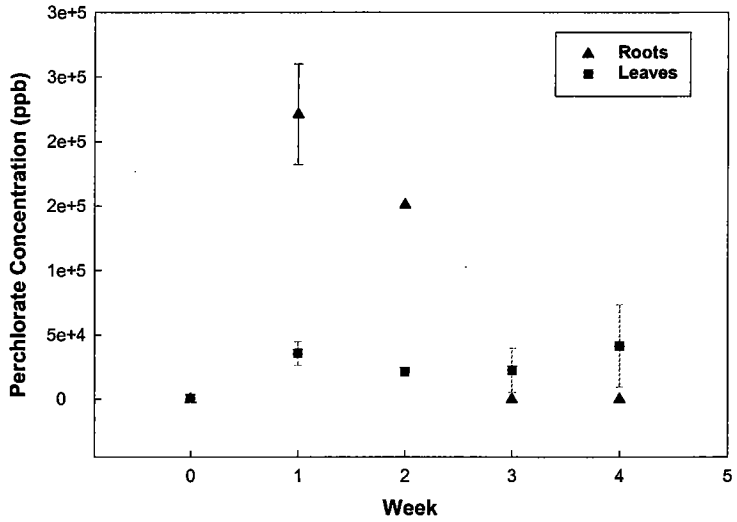

Figure 4. Perchlorate concentrations in roots and leaves from 4 week cucumber (*Cucumis sativus* L.) perchlorate uptake experiment in the presence of Hydrosol. Concentrations were calculated based on dry weight. Error bars represent one standard deviation of the mean.

In the 2 week uptake study with cucumber in the presence of DI water, there was also uptake of perchlorate into plants as indicated by both removal of perchlorate from planted sand and detection of perchlorate in leaves (**Table 3**). In addition, leaf concentrations of perchlorate were nearly an order of magnitude larger than perchlorate concentrations in plants fed Hydrosol. These results are suggestive of competition for uptake into the plant between perchlorate and other nutrients (likely nitrate).

Table 3. Uptake of perchlorate from sand into cucumber (*Cucumis sativus* L.) in the presence of DI water. Plant concentrations (mean ± SD) were calculated based on dry weight.

	Unplanted Sand Conc (ppb)	Planted Sand Conc (ppb)	Root Conc (ppb)	Leaf Conc (ppb)
Week 0	110 ± 5	110 ± 5	ND	ND
Week 1	85	16 ± 1	ND	118077±11280
Week 2	98 ± 4	15	ND	202425

ND = not detected

An analysis of the sand data from these two experiments (Hydrosol vs. DI water) indicated that perchlorate was removed from sand quicker in the experiment with DI water than in the experiment with Hydrosol (**Figure 5**). This result supports our previous conclusion on the competition for uptake of perchlorate.

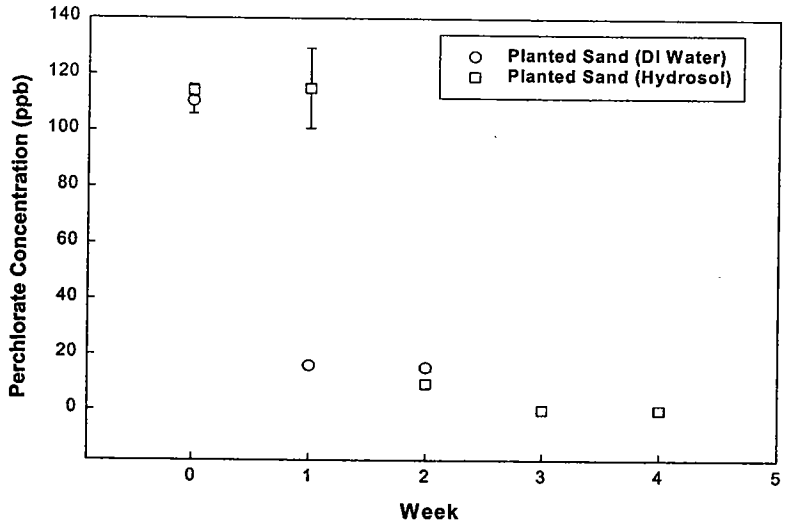

Figure 5. A comparison of sand concentrations in the 2 week cucumber perchlorate uptake experiment with DI water and the 4 week cucumber perchlorate uptake experiment with Hydrosol. Error bars represent one standard deviation of the mean.

Perchlorate was not detected in roots of plants fed DI water (**Figure 6**). This results is not because perchlorate was not taken up into roots. Rather, perchlorate was taken up into roots and translocated to leaves before the end of the first week. This conclusion is supported by the leaf data presented in **Figure 7**. Perchlorate concentrations in the leaves and stems had a sharp increase from week 0 to week 2 in the experiment with DI water. At week 2, perchlorate concentrations in cucumber leaves and stems fed DI water was more than 4-fold higher than perchlorate concentrations in cucumber leaves and stems fed Hydrosol.

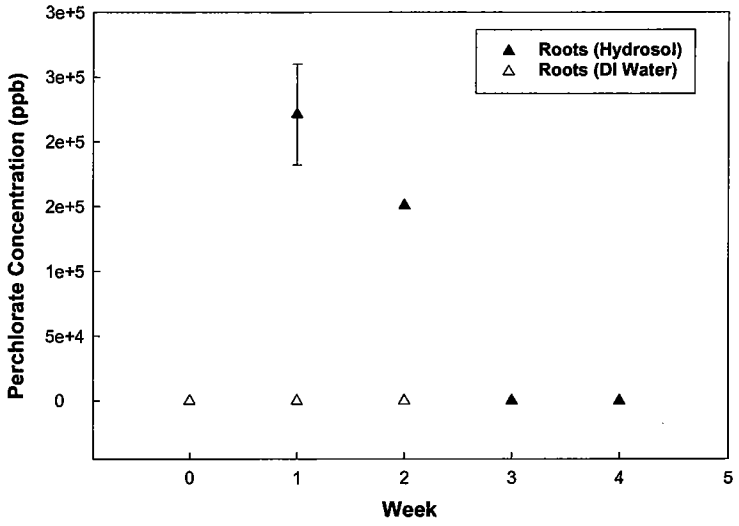

Figure 6. A comparison of root concentrations in the 2 week cucumber perchlorate uptake experiment with DI water and the 4 week cucumber perchlorate uptake experiment with Hydrosol. Root concentrations were based on dry weights. Error bars represent one standard deviation of the mean.

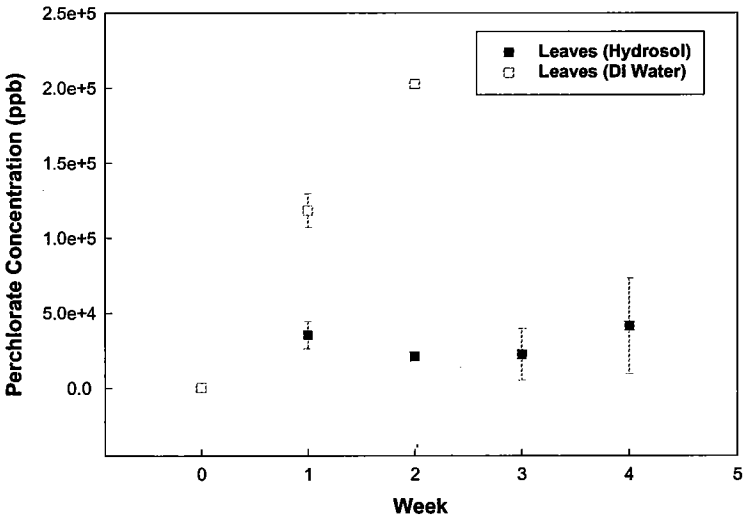

Figure 7. A comparison of leaf concentrations in the 2 week cucumber perchlorate uptake experiment with DI water and the 4 week cucumber perchlorate uptake experiment with Hydrosol. Leaf concentrations were based on dry weights. Error bars represent one standard deviation of the mean.

In order to further elucidate the competition phenomenon, we measured perchlorate uptake at different Hydrosol levels rather than simply 100% and 0% (DI water) Hydrosol. There was uptake of perchlorate into the leaves of cucumber plants during the 4 week uptake study in a solution containing 50:50 Hydrosol:Milli-Q water

(**Table 4, Figures 8 and 9**). In addition, similar results were obtained when the uptake experiment was conducted in a solution of 25:75 Hydrosol:Milli-Q water (**Table 5, Figures 10 and 11**).

Table 4. Uptake of sodium perchlorate from sand into cucumber (*Cucumis sativus* L.) in the presence of Hydrosol:Milli-Q water (50:50). Duration of the experiment was 4 weeks. The plant concentrations (mean ± SD) were calculated based on dry weight.

	Unplanted Sand Conc (ppb)	Planted Sand Conc (ppb)	Root Conc (ppb)	Leaf Conc (ppb)
Week 0	104 ± 2	104 ± 2	ND	ND
Week 0.5	102 ± 10	102 ± 10	0	ND
Week 1	94	54 ±19	313891 ± 51375	98049 ± 44689
Week 1.5	100	43 ±17	ND	159903 ± 33529
Week 2	106	26 ± 8	ND	267739 ± 19193
Week 3	65	ND	ND	126518 ± 56962
Week 4	85	ND	ND	139058 ± 20553

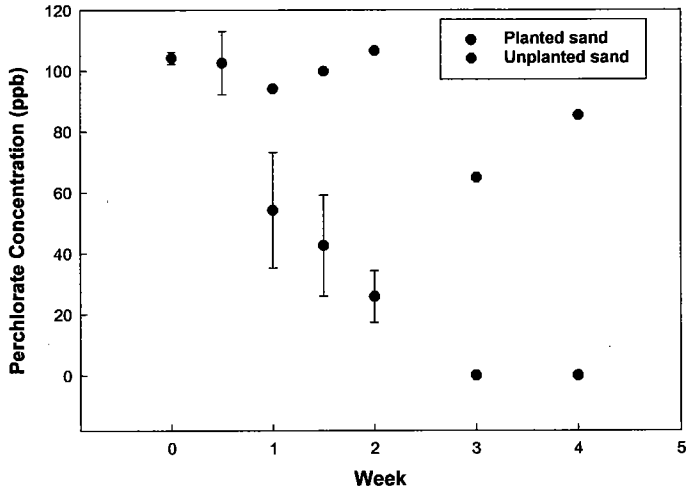

Figure 8. Perchlorate concentrations in sand from 4 week cucumber (*Cucumis sativus* L.) perchlorate uptake experiment in the presence of Hydrosol:Milli-Q water (50:50). Error bars represent one standard deviation of the mean.

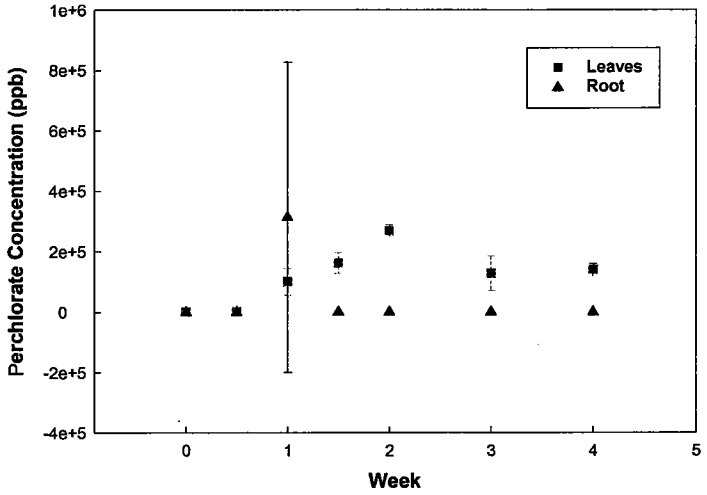

Figure 9. Perchlorate concentrations in roots and leaves from 4 week cucumber (*Cucumis sativus* L.) perchlorate uptake experiment in the presence of Hydrosol:Milli-Q water (50:50). Concentrations were calculated based on dry weight. Error bars represent one standard deviation of the mean.

Table 5. Uptake of sodium perchlorate from sand into cucumber (*Cucumis sativus* L.) in the presence of Hydrosol:Milli-Q water (25:75). Plant concentrations (mean ± SD) were calculated based on dry weight.

	Unplanted Sand Conc (ppb)	Planted Sand Conc (ppb)	Root Conc (ppb)	Leaf Conc (ppb)
Week 0	108 ± 2	108 ± 2	ND	ND
Week 0.5	107 ± 2	107 ± 2	ND	ND
Week 1	99 ± 7	61 ±18	5495 ± 77771	127398 ± 31067
Week 1.5	109	30 ±13	ND	201549 ± 22315
Week 2	108	25 ± 6	9416 ± 18833	190623 ± 16033
Week 3	72	2 ± 5	ND	314375 ± 55700
Week 4	102	ND	ND	219093 ± 32953

ND = not detected

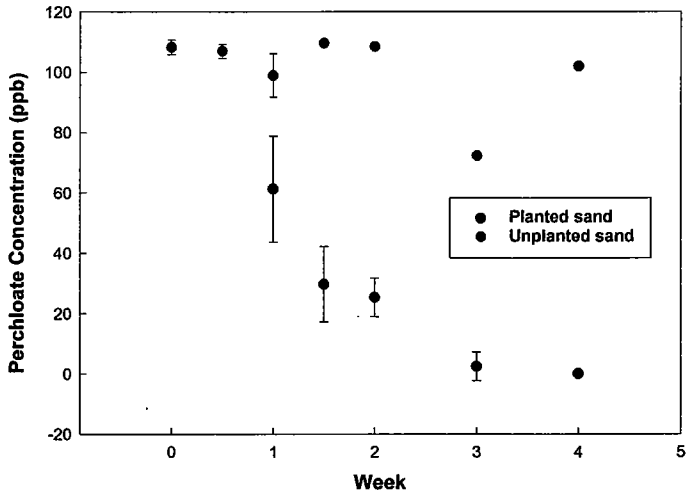

Figure 10. Perchlorate concentrations in sand from 4 week cucumber (*Cucumis sativus* L.) perchlorate uptake experiment in the presence of Hydrosol:Milli-Q water (25:75). Error bars represent one standard deviation of the mean.

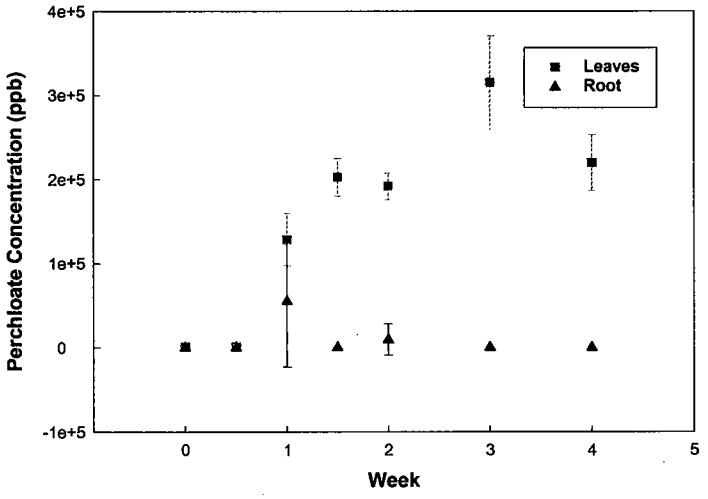

Figure 11. Perchlorate concentrations in roots and leaves from 4 week cucumber (*Cucumis sativus* L.) perchlorate uptake experiment in the presence of Hydrosol:Milli-Q water (25:75). Plant concentrations were calculated based on dry weight. Error bars represent one standard deviation of the mean.

A summary comparison was conducted to determine differences in perchlorate uptake in cucumber with respect to varying ratios of Hydrosol to water. From these comparisons, it was clear that perchlorate concentrations in sand decreased quickest in the DI water system, and slowest in the 100% Hydrosol system. Perchlorate concentrations in sand from experiments conducted at the other ratios of Hydrosol to water decrease at a rate in between those of pure Hydrosol and pure water (**Figure 12**). Leaf and root concentrations were also compared among the experiments discussed above. Consistent with the sand data, perchlorate uptake was the greatest when Hydrosol was absent or limited (**Figures 13 and 14**).

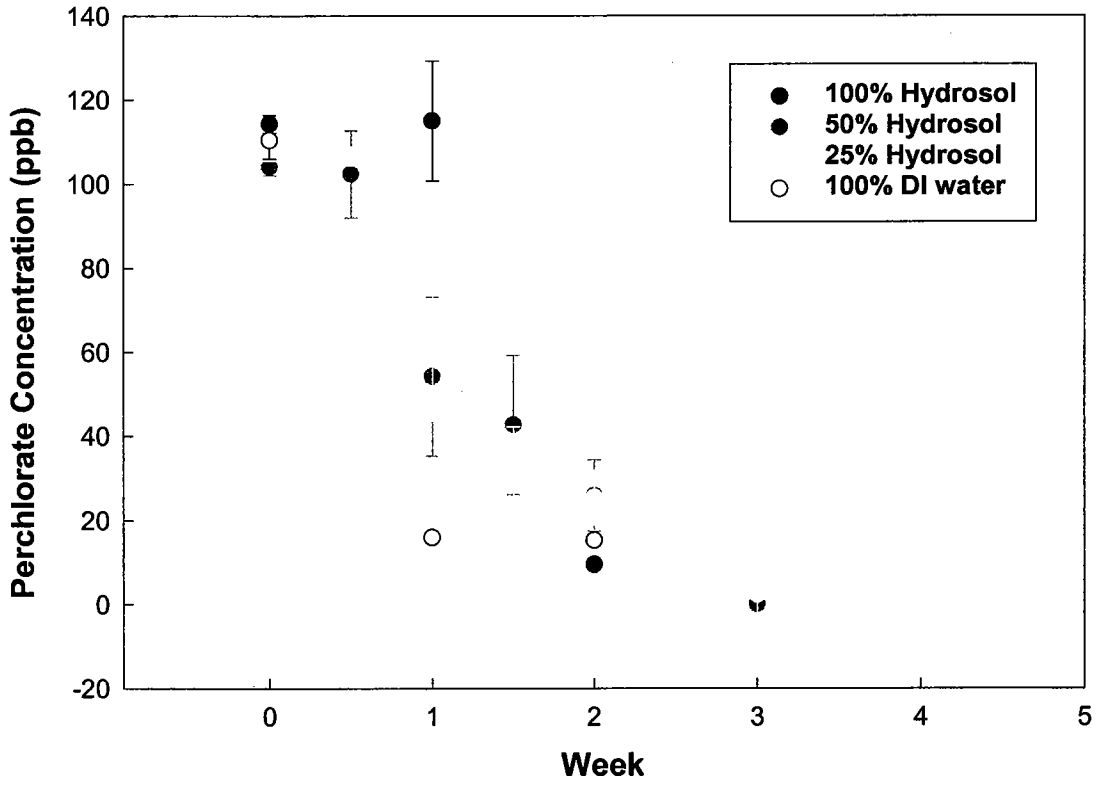

Figure 12. A comparison of sand concentrations in 4 cucumber perchlorate uptake experiments in the presence of varying ratios of Hydrosol to water. Error bars represent one standard deviation of the mean.

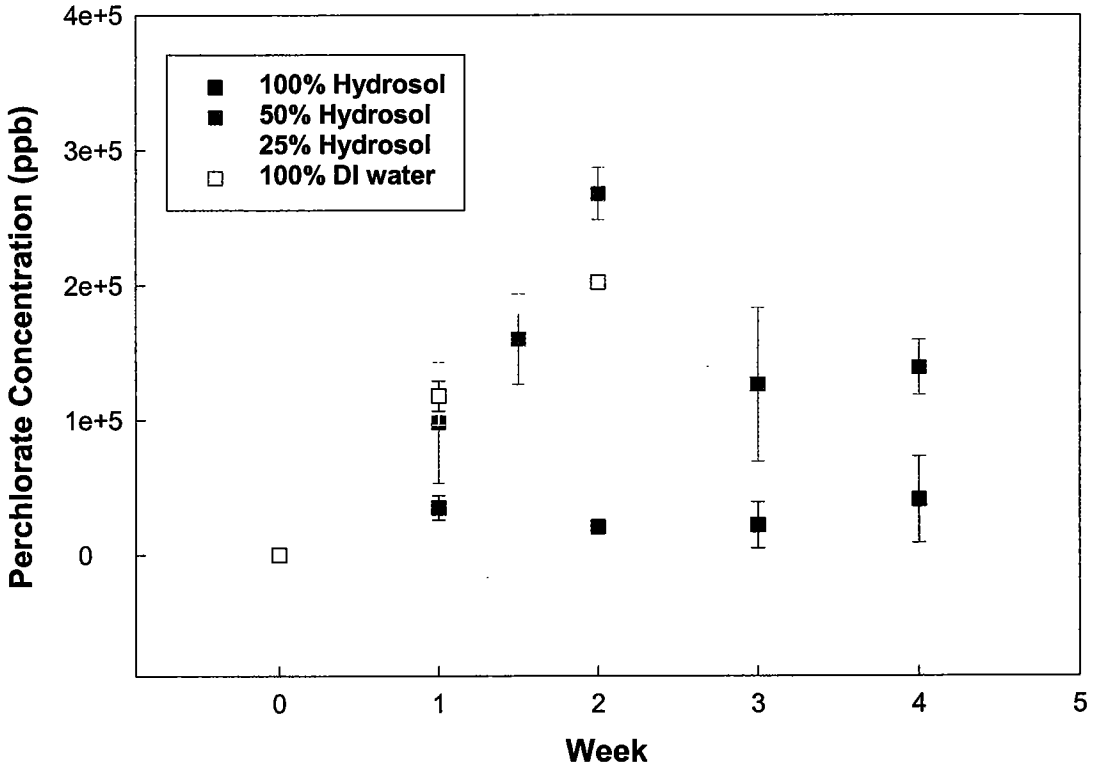

Figure 13. A comparison of leaf concentrations of 4 cucumber perchlorate uptake experiments in the presence of varying ratios of Hydrosol to water. Error bars represent one standard deviation of the mean.

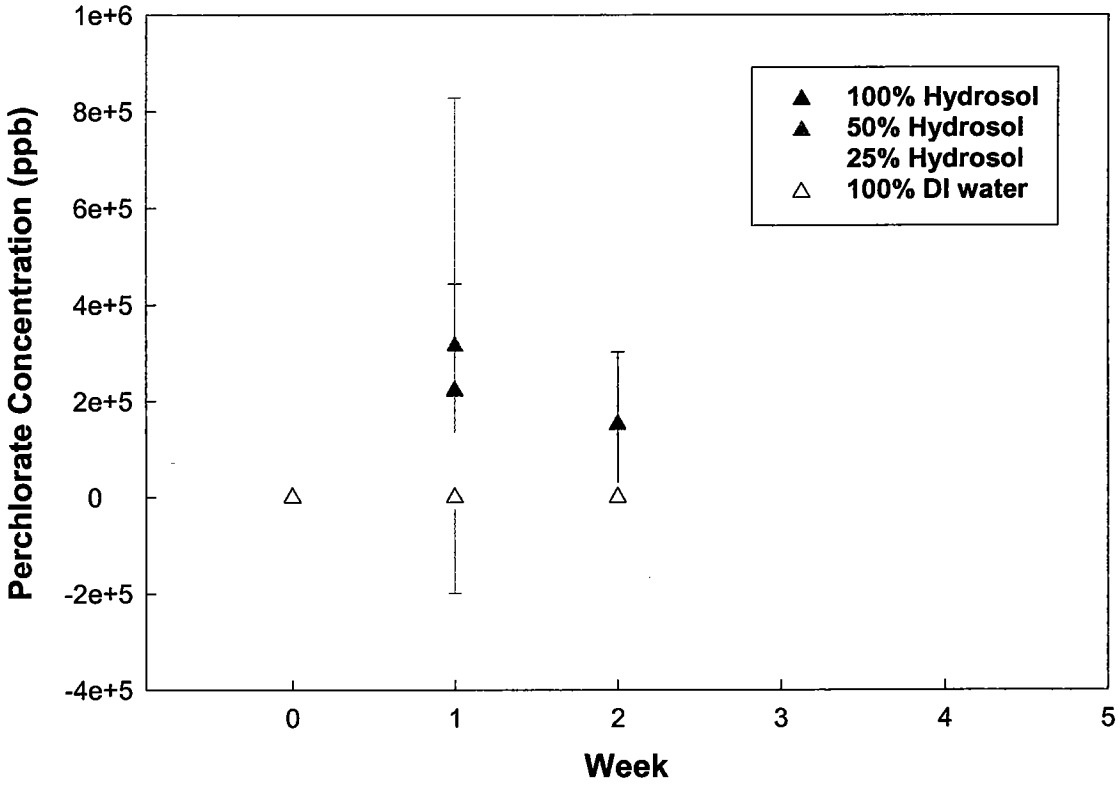

Figure 14. A comparison of root concentrations from 4 cucumber perchlorate uptake experiments in the presence of varying ratios of Hydrosol to water. Error bars represent one standard deviation of the mean.

In the 8 week perchlorate uptake study with cucumber, treatment cups were respiked with 100 ppb perchlorate at week 4. Sand concentrations remained low (at the next sampling period) despite the respiking, indicating that perchlorate uptake is quite rapid (**Table 6, Figure 15**). Leaf concentrations peaked at week 3 at nearly 200 ppm and then curiously decreased slightly (**Figure 16**). After the samples were respiked, leaf concentrations increased and peaked again at week 6 (200 ppm). It is likely that this 200 ppm threshold is a result of complete removal of available perchlorate in the system rather than reaching a maximum perchlorate burden and subsequent excretion. We tested the evapotranspiration water that accumulated on the sides of the cups (not present in the unplanted controls). This water contained perchlorate, but this may not necessarily be an indication that the plant is excreting perchlorate.

Table 6. Uptake of sodium perchlorate from sand into cucumber (*Cucumis sativus* L.) in the presence of Hydrosol during an 8 week study. Plant concentrations (mean ± SD) were calculated based on dry weight.

	Unplanted Sand Conc (ppb)	Planted Sand Conc (ppb)	Root Conc (ppb)	Leaf Conc (ppb)
Week 0	106 ± 4	106 ± 4	ND	ND
Week 0.5	96 ± 3	96 ± 3	ND	ND
Week 1	94 ± 5	55 ± 4	ND	81606 ± 13548
Week 1.5	128	41 ± 9	19515 ± 39031	114389 ± 7192
Week 2	85	57 ± 19	33664 ± 67327	99913 ± 13830
Week 3	96	22 ± 16	ND	142051 ± 10501
Week 4	62 ± 3	6 ± 5	ND	101659 ± 45349
Week 5	184	36 ± 22	ND	119380 ± 61927
Week 6	164	47 ± 22	ND	146559 ± 9151
Week 7	238	45 ± 20	ND	102079 ± 9322
Week 8	149	20 ±17	ND	79780 ± 31284

ND = not detected

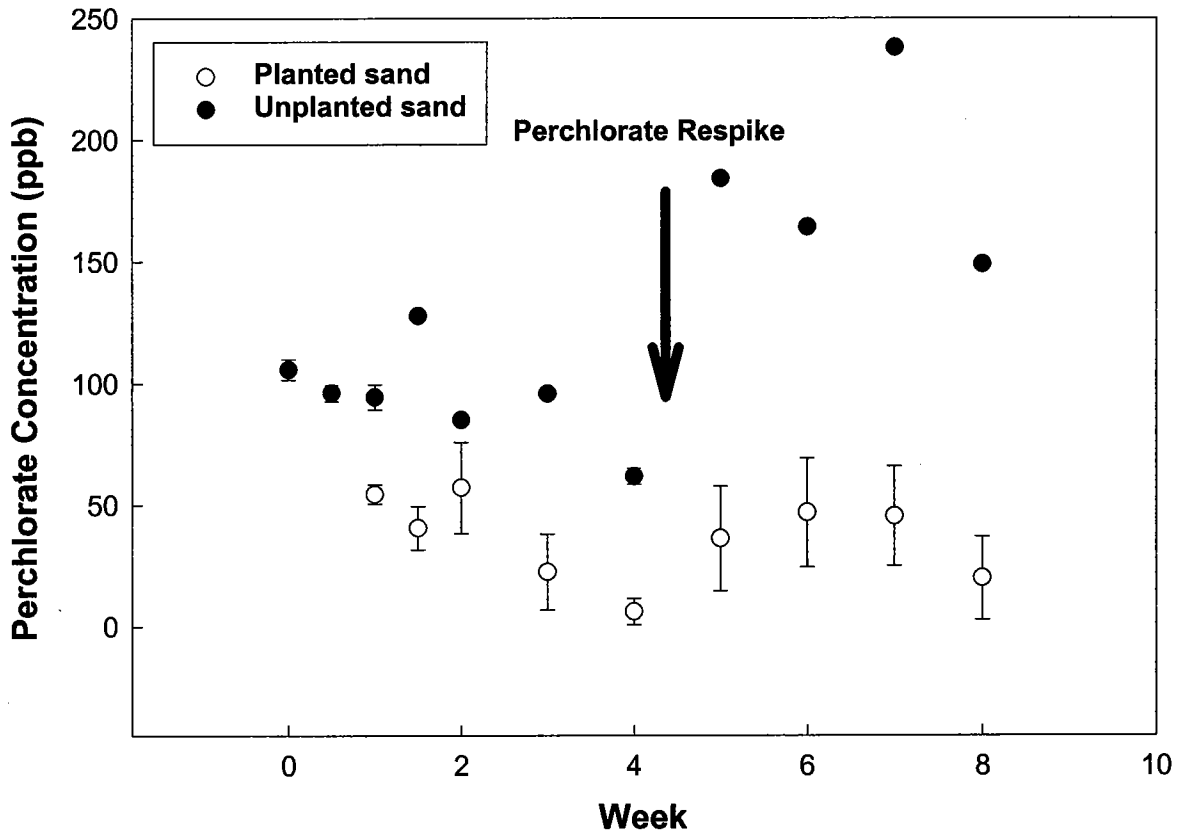

Figure 15. Perchlorate concentrations in sand from 8 week cucumber (*Cucumis sativus* L.) perchlorate uptake experiment in the presence of Hydrosol. Error bars represent one standard deviation of the mean.

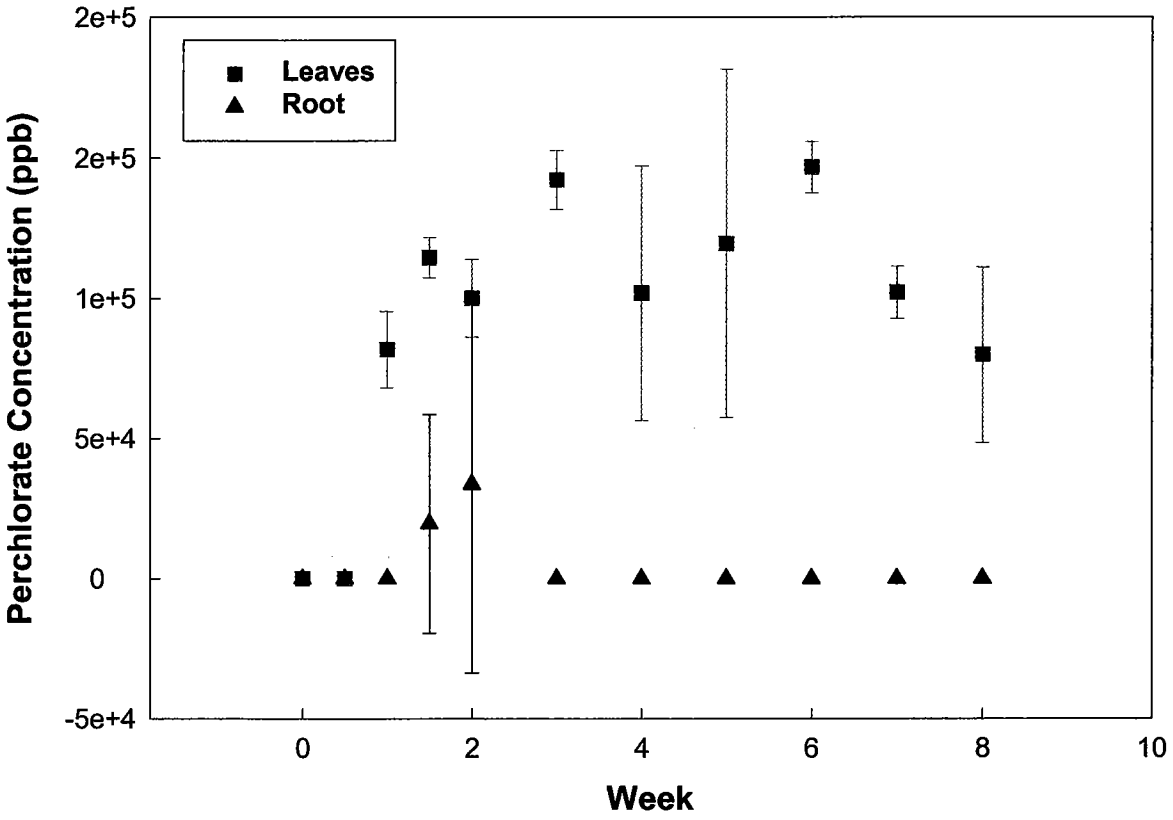

Figure 16. Perchlorate concentrations in roots and leaves from 8 week cucumber (*Cucumis sativus* L.) perchlorate uptake experiment in the presence of Hydrosol. Concentrations were calculated based on dry weight. Error bars represent one standard deviation of the mean.

16.2.2 Duckweed

There appeared to be some uptake of perchlroate in duckweed grown solely in Hydrosol versus those grown in DI water or in a mixture of DI water and Hydrosol (**Table 7**), contrary to the findings with terrestrial plants. Uptake of perchlorate into *Myriophyllum* sp. was also examined under conditions similar to the duckweed tests. There was no uptake of perchlorate observed in this plant.

Table 7. Concentrations of perchlorate (mean ρ SD) from 4 studies of perchlorate uptake into duckweed (*Lemna minor*). All tests were conducted at a concentration of 100 ppb sodium perchlorate. Plant concentrations are based on wet weights.

Test	# Replicates	Conc in plants (ng/g)
Initial Uptake from Hydrosol	4	1713 ρ 205
Time course of uptake from Hydrosol		
Day 1	2	134 ρ 189
Day 3	1	291
Day 5	2	235 ρ 5
Day 10	2	79 ρ 30
Uptake from DI water	4	ND
Uptake from 50:50 Hydrosol:Water	4	ND

ND = not detected

16.2.3 Lettuce

Perchlorate uptake from sand into lettuce was consitent with our previous observations in cucumber (**Table 8**, **Figure 17**). Namely, perchlorate was rapidly removed from planted sand and taken up and translocated in lettuce.

Table 8. Uptake of perchlorate from sand into lettuce (*Lactuca sativa* L.) in the presence of Hydrosol. Plant concentrations (mean ± SD) were calculated based on dry weight.

	Unplanted Sand Conc (ppb)	Planted Sand Conc (ppb)	Root Conc (ppb)	Leaf Conc (ppb)
Week 0	69 ± 2	69 ± 2	ND	ND
Week 1	70	72 ± 2	ND	115656
Week 2	82	68 ± 8	ND	241268 ± 16642
Week 3	55	72 ± 19	ND	70314 ± 23193
Week 4	43	8 ±11	ND	75379 ± 32577
Week 5	79	ND	19256	20762 ± 6239
Week 6	71	ND	ND	31871 ± 14367

ND = not detected

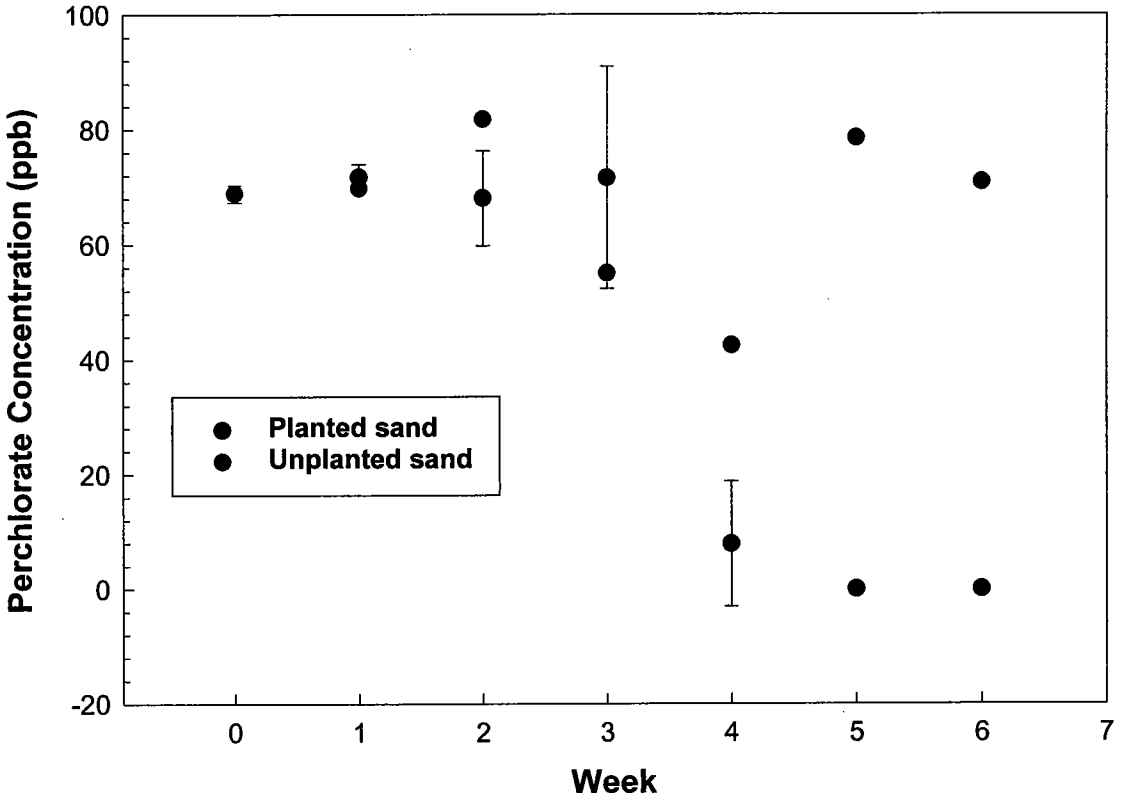

Figure 17. Perchlorate concentrations in sand from 6 week lettuce (*Lactuca sativa* L.) perchlorate uptake experiment in the presence of Hydrosol. Error bars represent one standard deviation of the mean.

Leaf concentrations in weeks 5 and 6 decreased from the peak concentration at week 4 (**Figure 18**) and perchlorate concentrations in rinse water increased significantly compared with rinse waters of weeks 3 and 4. This may be due to expiration of perchlorate in water to the surface of the leaf or excretion of perchlorate by the plant.

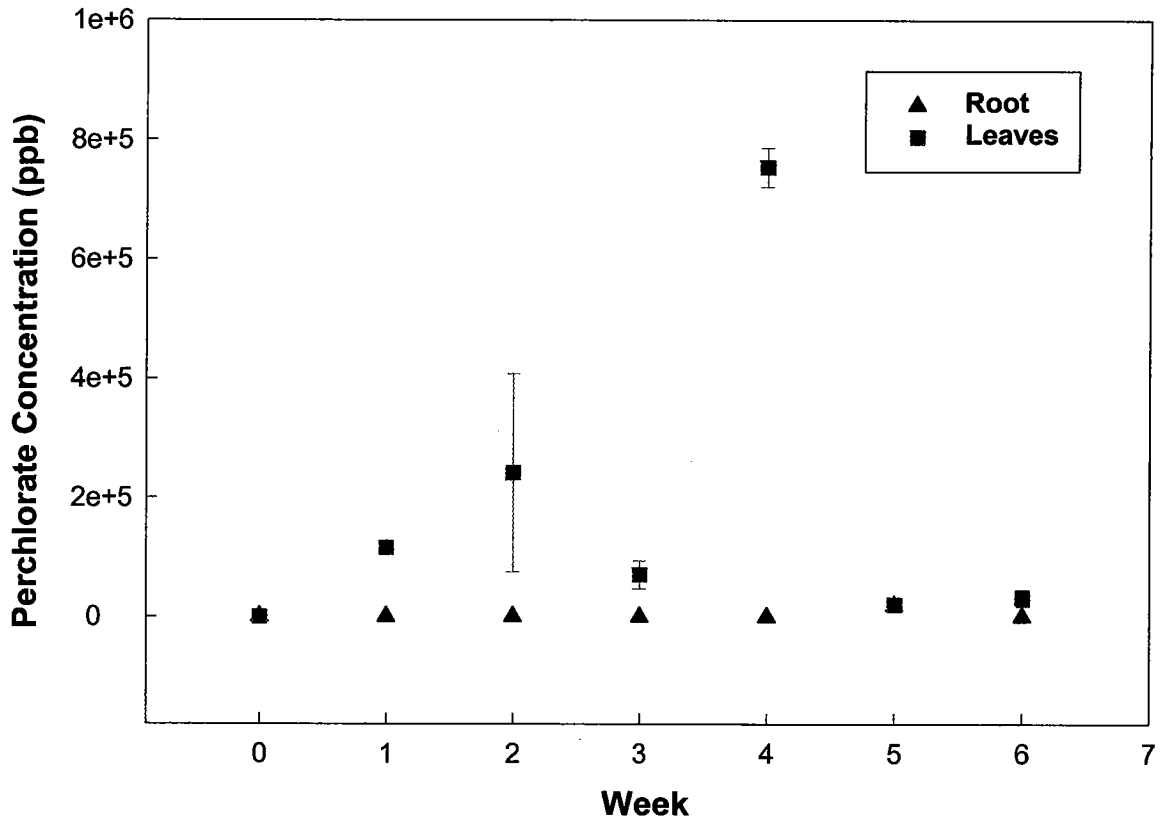

Figure 18. Perchlorate concentrations in roots and leaves from 6 week lettuce (*Lactuca sativa* L.) perchlorate uptake experiment in the presence of Hydrosol. Plant concentrations were calculated based on dry weight. Error bars represent one standard deviation of the mean.

16.2.4 Soybean

In the 3 week uptake study of sodium perchlorate into soybeans in a recirculating hydroponic system, there was uptake of perchlorate into soybeans (**Table 9**). Root and leaf uptake factors were also determined.

Table 9. Uptake of perchlorate into soybeans (*Glycine max*) in a recirculating hydroponic system. Plant concentrations (mean ± SD) were calculated based on dry weight. There were two replicates in the 0 ppb treatment group and 4 four replicates in both the 25 ppb and 100 ppb treatment groups.

Perchlorate treatment	Perchlorate in root (ppb)	Perchlorate in leaves and stems (ppb)	Root uptake factor	Leaves and stems uptake factor
0 ppb	0	0	0	0
25 ppb	3112 ± 1143	5611 ± 5661	125	224
100 ppb	9925 ± 3109	7290 ±6634	99	73

From the 4 week study of perchlorate uptake from sand into soybeans in the presence of Hydrosol, perchlorate concentrations decreased in the sand with the presence of plant and increased in root and leaf concentrations (**Table 10, Figures 19 and 20**) consistent with our previous observations. Root concentrations increased in the first two weeks and decreased with the sand at weeks 3 and week 4 (**Figures 19 and 20**).

Table 10. Uptake of sodium perchlorate from sand into soybean (*Glycine max*) in the presence of Hydrosol. Plant concentrations (mean ± SD) were calculated based on dry weight.

	Unplanted Sand Conc (ppb)	Planted Sand Conc (ppb)	Root Conc (ppb)	Leaf Conc (ppb)
Week 0	12 ± 3	128 ± 3	0	0
Week 1	114 ± 9	83 ± 8	9309 ± 18618	12995 ± 6313
Week 2	77 ± 3	11 ±10	28838 ± 22590	15323 ± 3584
Week 3	62 ± 5	8 ± 2	0	17774 ± 3949
Week 4	67 ± 4	18 ± 16	0	14497 ± 3050

ND = not detected

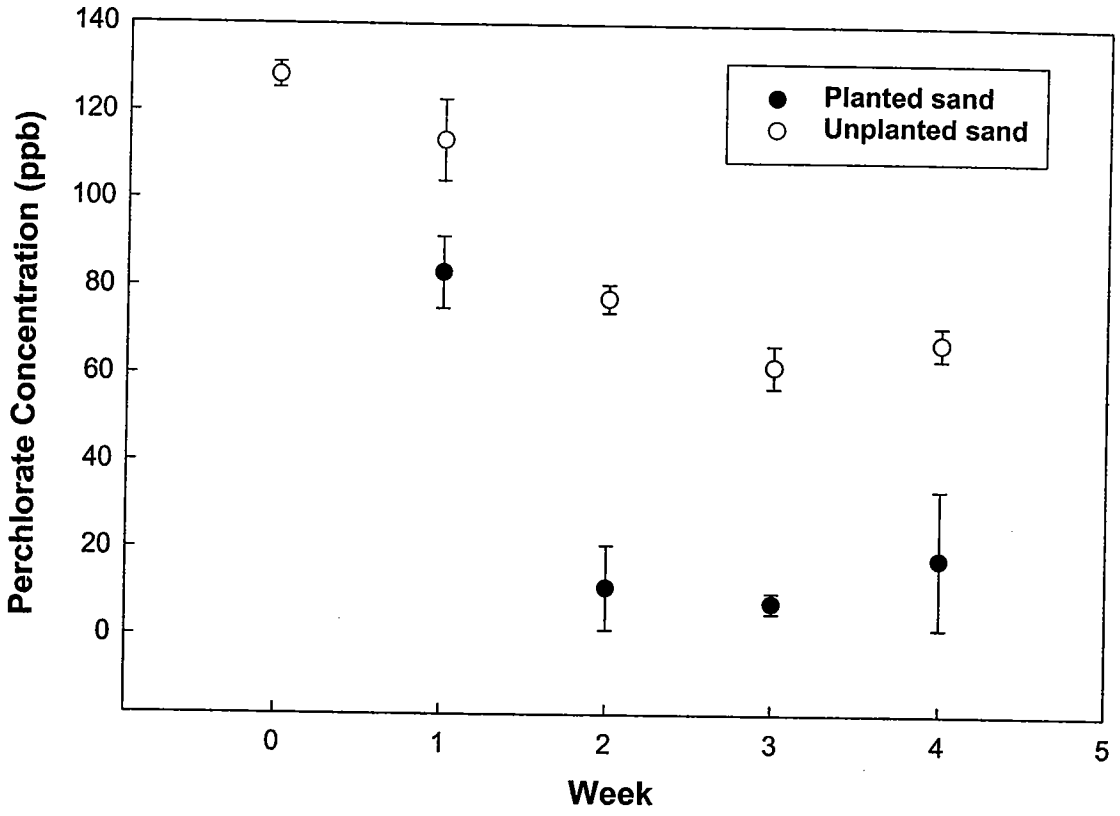

Figure 19. Perchlorate concentrations in sand from 4 week soybean (*Glycine max*) perchlorate uptake experiment in the presence of Hydrosol. Error bars represent one standard deviation of the mean.

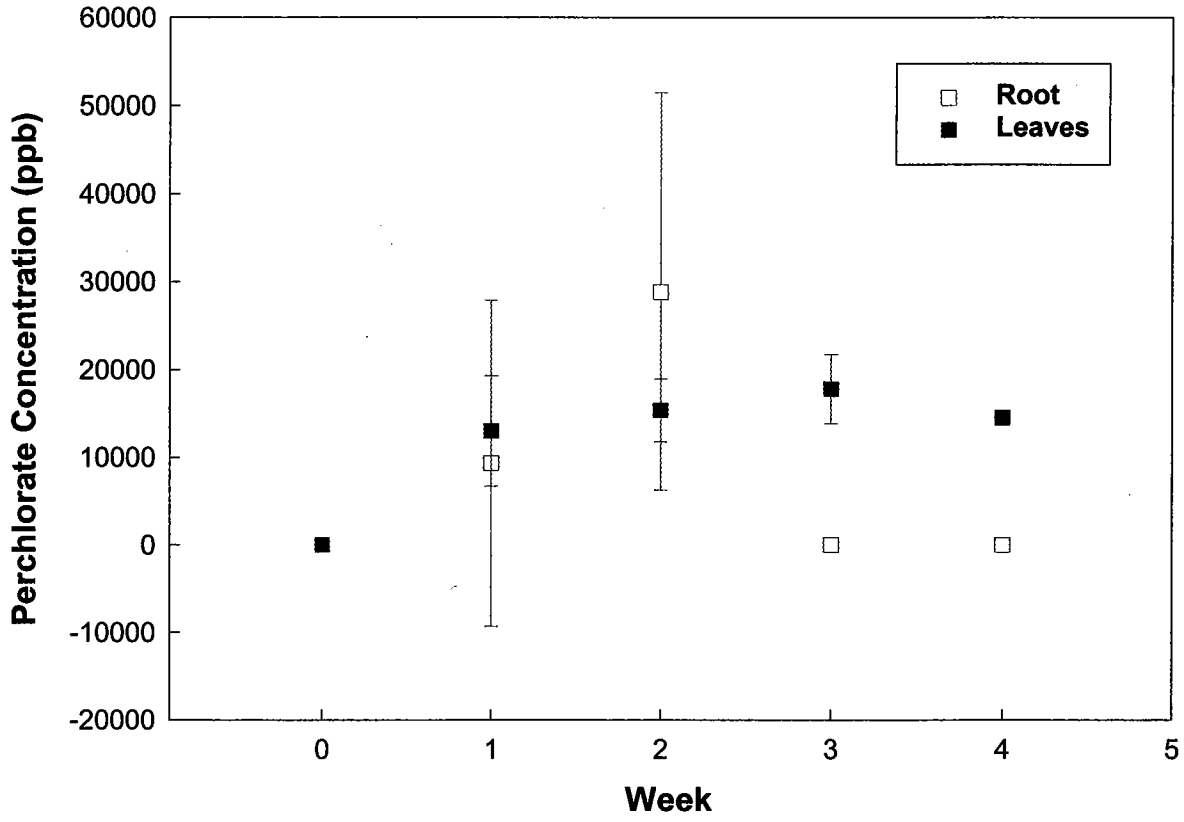

Figure 20. Perchlorate concentrations in roots and leaves from 4 week soybean (*Glycine max*) perchlorate uptake experiment in the presence of Hydrosol. Error bars represent one standard deviation of the mean.

16.3 Field Data

Several plant samples from the Longhorn Army Ammunition Plant (LHAAP) had detectable levels of perchlorate. Perchlorate was found in willow, fern, juncus (INF), Bahia grass (Burning Ground (BG)), algae (25C) and bullrush plants as well as in soils surrounding these plant species. Plant uptake factors were calculated for these data (**Table 11**). Although perchlorate was detected in INF pond in September 2000, there was no perchlorate detected in August 2001 due to the remediation effort established earlier that year.

Table 11. Perchlorate concentrations in vegetation and soil/water samples from Building 25C and the INF Pond at LHAAP. Samples were collected in August 2001 unless otherwise noted.

Plant	Plant concentration (ppb)	Soil/water concentration (ppb)	Plant uptake factor
Willow	19755	129	153
Grass	ND	ND	NA
Fern	2589	54	48
Juncus (25C)	2350	76	31
Juncus (INF)	ND	ND	NA
Bahia grass (BG)	4849	NA	NA
Algae (25C)	249	NA	NA
Bullrush above water*	7622 ±1460	279 ± 305**	27
Bullrush below water*	4451 ± 2241	279 ± 305**	16
Bullrush roots*	844 ± 409	279 ± 305**	3

*Collected on September 13, 2000.
**Water collected in November 1999.

17.0 STUDY RECORDS AND ARCHIVE:

Study Records will be maintained at The Institute of Environmental and Human Health (TIEHH) Archive for a minimum of one year after study completion date.

18.0 REFERENCES:

Edward TU, Matthew LM, Catherine AK, Stephanie KB. 2000. Perchlorate uptake by salt cedar (*Tamarix ramosissima*) in the Las Vegas Wash riparian ecosystem. The Science of the Total Environment 256: 227-232.

Susarla STB, Harvey G and McCutcheon SC. 2000. Phytotransformations of perchlorate contaminated waters. J. Environmental Toxicology 21:1055-1065.

U.S. Environmental Protection Agency (USEPA). 1998. Perchlorate Environmental Contamination: Toxicological Review and Risk Characterization Based on Emerging Information. Office of Research and Development, Washington, DC. NCEA-1-0503.

A FINAL REPORT

ENTITLED

UPTAKE OF AMMONIUM PERCHLORATE AND THYROID STATUS IN NATIVE FISH

STUDY NUMBER:	FISH 01-01
SPONSOR:	Strategic Environmental Research and Development Program (SERDP) 1155 Herndon Parkway, Suite 900 Herndon, Virginia 20170
CONTRACT ADMINISTRATOR:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
TESTING FACILITY:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, Texas 79409-1163
TEST SITE:	The Institute of Environmental and Human Health Texas Tech University Box 41163 Lubbock, Texas 79409-1163
ANALYTICAL TEST SITE:	The Institute of Environmental and Human Health Texas Tech University / TTU Health Sciences Center Box 41163 Lubbock, Texas 79409-1163
RESEARCH INITIATION:	03/01/2001
RESEARCH COMPLETION:	02/09/2002

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List of Tables and Figures

GOOD LABORATORIES PRACTICES STATEMENT

Project FISH 01-01, entitled "Uptake of Ammonium Perchlorate and Thyroid Status in Native Fish", was performed whenever possible in the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989

Submitted By:

Christopher Theodorakis, Ph.D

3/28/02
Date

QUALITY ASSURANCE STATEMENT

This study was conducted under the Institute of Environmental and Human Health's Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Any changes in protocol and SOPs were documented in writing and signed by the study director.

The Quality Assurance Officer verbally notified the Study Director of all findings at the time of the inspection. Written audit reports were also submitted to the Study Director and Test Facility Management. Audits were performed for the following phases of the project:

Auditable Research Phase/Activity	Audit Dates		Date written report submitted to Study Director	Date written report submitted to Management
	Start	End		
Final Report and Raw Data Review	03/01/02	03/12/02		

Submitted By:

Ryan Bounds
Quality Assurance Manager

3/28/02
Date

1. **DESCRIPTIVE STUDY TITLE:**
Uptake of Ammonium Perchlorate and Thyroid Status in Native Fish

2. **STUDY NUMBER:** FISH 01-01

3. **SPONSOR:**
Strategic Environmental Research
and Development Program (SERDP)
1155 Herndon Parkway, Suite 900
Herndon, Virginia 20170

4. **TESTING FACILITY NAME AND ADDRESS:**
The Institute of Environmental and Human Health
Texas Tech University
Box 41163
Lubbock, Texas 79409-1163

5. **PROPOSED EXPERIMENTAL START & TERMINATION DATES:**
Start: 03/01/2001
Termination: 02/09/2002

6. **KEY PERSONNEL:**
Christopher Theodorakis, Study Director
Todd Anderson, Analytical Chemist
Ryan Bounds, Quality Assurance Officer
Ron Kendall, Principal Investigator
Carrie Bradford, Technician
Jacques Rinchard, Technician
Irene Bier, Technician

7. **STUDY SUMMARY:**
Mosquitofish were exposed to 0, 0.1, 1, 10, 100, and 1000 ppm sodium perchlorate for 2, 10, and 30 days. Following laboratory exposure fish were euthanized and preserved in liquid nitrogen for body burden analysis or in Bouin's fixative for thyroid histology. Perchlorate was taken up in the fish in a dose response manner, but did not bioaccumulate. Perchlorate was also demonstrated to induce an increase of the epithelial cell height. Perchlorate induced hyperplasia of the thyroid follicle epithelium.

Catfish were exposed to 100 ppm sodium perchlorate for 5 days. Following the exposure, the catfish were euthanized and dissected and the kidney, liver, gonad, gill, GI tract, head, and fillet were removed and preserved in liquid nitrogen. The head was found to have the highest concentration of perchlorate, with a large amount also present in the fillet. Low concentrations of perchlorate were present in the other tissues analyzed.

8. STUDY OBJECTIVES / PURPOSE:

The objectives of this study were to determine kinetics of uptake and relative tissue distributions of sodium perchlorate in native fish species and to determine the effects of perchlorate as reflected by thyroid histology.

9. TEST MATERIALS:

Test Chemical: Sodium Perchlorate
CAS Number : 7601-89-0
Characterization: Determination of concentration in environmental samples.
Source: EM Science

Reference Chemical: Ultrapure water with added sea salts ("Instant Ocean®")
CAS Number : NA
Characterization: Determination of pH and conductivity.
Source: City tap water that has been run through reverse osmosis and a de-ionizer to convert it to ultrapure water. 60 mg/L sea salts was added.

10. JUSTIFICATION OF TEST SYSTEM:

Ionic perchlorate alters thyroid homeostasis in fishes and amphibians as well as other vertebrates (Miranda et al., 1996; Manzon and Youson, 1997). Because of the important role played by hormones in animal development and reproduction, endocrine disruption is likely to lead to serious impairments in growth, reproductive fitness, and consequently, fish and wildlife population stability as well as human health.

11. TEST ANIMALS:

Species: *Gambusia holbrooki*, mosquitofish; *Ictalurus punctatus*, channel catfish
Strain: Wild
Age: Adults.
Number: Approximately 1350 mosquitofish, and 20 catfish
Source: Purchased from hatcheries

12. PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:

The test system consisted of laboratory exposures constructed according to the experimental design described below. Identity of all fish was confirmed in the laboratory by visual inspection before tests were begun. Aquaria were labeled with the aquaria number, species name, animal use protocol number, project number, test system, date of exposure and date of collection, concentration, and person responsible.

13. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:

Mosquitofish were exposed to five concentrations plus zero concentration or control and catfish were exposed to one concentration of sodium perchlorate. Fish were placed into precleaned aquaria. Aquaria were cleaned by washing each aquarium according to SOP AQ-1-02 "Cleaning Glassware and Aquaria for Perchlorate Assays". For exposures, aquaria were located on shelves capable of supporting such weight. Each shelf held six

20L aquaria (for the mosquitofish and catfish). The experimental design consisted of a randomized block design, with each shelf constituting a block. The arrangement of the aquaria within each block was randomized in order to avoid effects due to gradients in light, temperature, volatile chemicals in the laboratory, etc. Determination of the arrangement of the aquaria or beakers within each block was done by rolling dice. Each block contained one aquarium of each treatment. Fish were placed in the aquaria in random order, within blocks, using the procedure described below.

For the mosquitofish, each block consisted of 6 aquaria, each with 15 fish. There were 5 blocks, for a total of 450 fish. All fish were placed in one aquaria. Within each block, each aquarium was assigned a number from 1-6. A random number generator was used to randomly order the numbers 1-6, and this was done 15 times: e.g., 341256, 465123, 564312, 435126, 345126, 163452, 234516, 132465, 235461, 213465, 623415, 342156, 623415, 614325, 534261. These numbers were generated with a random number generator. The fish were placed into the 6 aquaria in the 1st block according to this list of numbers. For example, one fish was placed into aquarium 3, then aquarium 4, aquarium 1, aquarium 2, aquarium 5 and finally aquarium 6. A second fish was placed in each aquarium in the order 465123. A third fish was placed in each aquarium in the order 564312, and so on, until there were 15 fish in each aquarium. This was done for the other 4 blocks in the experiment.

Only females were used for the mosquitofish exposures in order to avoid sex effects. Mosquitofish that weigh approximately 0.7-1.2 g, and catfish that weigh approximately 120-150 g, were used for these experiments.

14. METHODS

14.1 Test System acquisition, quarantine, acclimation

Fish were obtained from fish hatcheries. Upon arrival at the lab, they were treated with commercially available antibiotics for 5 days, as instructed by the manufacturer. After five days, any debris at the bottom of the tank and 1/3 of the tank water was removed using a siphon hose and replaced with fresh water. Fresh water consisted of reverse osmosis (RO) water supplemented with 60 mg/L Instant Ocean sea salts. Fresh water was replaced in each tank by siphon from a reservoir (e.g., 70 gallon aquarium). Every other day, debris at the bottom of the tank was cleaned by suction. Water was continuously aerated and filtered using mechanical and biological filtration. Animal husbandry was conducted according to SOP AQ-1-08, "General Fish Husbandry" and SOP AQ-1-09 "Mosquitofish *Gambusia spp.* Husbandry". Total acclimatization period was a minimum of one week. Once acclimated, fish were exposed to sodium perchlorate dissolved in ultrapure water. Mosquitofish were fed commercial flake goldfish food at the rate of 5 mg per gram of fish, on a daily basis. Catfish were fed pelleted food at the same rate.

14.2 Test Condition Establishment

Exposures were begun after fish had become acclimatized.

14.3 **Test Material Application**

Mosquitofish were placed into aquaria containing various concentrations of sodium perchlorate, with 15 fish per aquarium and 5 replicate aquaria per treatment. Catfish were placed in aquaria containing 100 ppm sodium perchlorate, with one fish per aquaria and twenty replicate aquaria. Stock solutions of 1 g/L, 10 g/L, and 100 g/L sodium perchlorate in ultrapure water were used to dose the fish. The mosquitofish and catfish aquaria were filled with 15 L reconstituted fresh water and an appropriate amount of stock solution was added according to the desired concentration of the aquarium water. Every other day, debris was cleaned out of the aquaria and 1/3 of the water was replaced in each tank with reconstituted fresh water, and perchlorate stock solution was added to maintain the desired concentration.

Rates/concentrations: Mosquitofish were exposed to 0, 0.1, 1.0, 10, 100, and 1000 ppm sodium perchlorate in water and catfish were exposed to 100 ppm sodium perchlorate in water.

Frequency: Five replicate tanks of each concentration were continually exposed 2, 10, and 30 days (i.e., 6 concentration x 3 exposure periods = 18 treatments in all for mosquitofish). Because of the time involved in euthanization and preservation of the fish at the end of the exposure, two replicates were dosed on the first day of the experiment, two replicates were dosed on the second day of the experiment, and the last replicate was dosed on the third day of the experiment. Euthanization of fish was also staggered so that all fish were exposed for the same time period. The 2-day exposure experiment began on March 1, 2001 and ended on March 4, 2001. The 10-day exposure experiment began on March 7, 2001 and ended on March 19, 2001. The 30-day exposure experiment began on April 2, 2001 and ended on May 4, 2001. Twenty replicate tanks of 100 ppm sodium perchlorate were continually exposed for 5 days for the catfish. The catfish exposure began on July 15, 2001 and ended on July 21, 2001.

Route/Method of Application: Route was via dermal, oral, and respiratory exposure as the chemical was in the aquaria water.

Stock solutions for the study were mixed in precleaned glass containers as indicated in SOP AQ-1-02-01. Stock solutions were made by dissolving sodium perchlorate in ultrapure water, and the pH was adjusted to 7.4 (with 0.1N HCl or 0.1N NaOH, as appropriate). The appropriate amount of sodium perchlorate was weighed on a calibrated balance, and mixed into ultrapure water. The pH was checked on a calibrated pH meter

(calibrated according to SOP IN-4-06) and adjusted as above. After 1/3 of the aquarium water had been removed and replaced (see above instructions), an appropriate amount of stock solution was added to adjust the concentration to the original value.

For the mosquitofish, the 1 g/L stock solution was used for the 0.1 ppm (0.1 mg/L) treatment; the 10 g/L stock was used for the 1 ppm (1 mg/L) and 10 ppm (10 mg/L), treatments; and the 100 g/L stock was used for the 100 and 1000 ppm (100 and 1000 mg/L) treatments. For example, if there was 15L of water in a mosquitofish tank, 5L was removed and replaced every other day. For the aquaria that had the 1 ppm and 10 ppm, 0.0015 L (1.5 ml) of perchlorate stock solution was added to the 1 ppm aquarium and 0.015 L (15 ml) of perchlorate stock solution was added to the 10 ppm aquarium initially, and 0.5 mL and 5 mL of perchlorate stock solution, respectively, was added following water changes (e.g., (15L x 10 mg/L)/10,000 mg/L = 0.015 L, one-third of this was replaced after each water change). Stock solutions were measured out in an appropriate pipette.

For the catfish, the 100 g/L stock was used for all treatments. For these fish, 5 L of water was removed every other day. Thus, for the 100 ppm treatment, 15 mL of perchlorate stock solution was added initially, and 5 mL was added following each water change. Stock solutions were measured out in an appropriate pipette.

Justification for Exposure Route: Exposure by environmental waters is most appropriate because fish respire, ingest, and are dermally exposed to chemicals in the waters in which they live.

Exposure Verification: A sample of each concentration of treated water was collected at the beginning of the exposure period and when fish were removed from the aquarium for analysis. A sample was also collected on the seventh day of exposure during the 10 day exposure and once a week during the 30 day exposure. The concentration of perchlorate in the water was tested using ion chromatography. Water Sample analysis indicated that exposure concentrations were close to the target concentration. For the 2 day exposure, aquaria perchlorate concentrations ranged from 0-87 ppb in the control aquaria, 0.074-0.097 ppm in the 0.1 ppm aquaria, 0.74-0.88 ppm in the 1 ppm aquaria, 6.9-7.8 ppm in the 10 ppm aquaria, 68-126 ppm in the 100 ppm aquaria, and 688-1,137 in the 1,000 ppm aquaria. For the 10 day exposure, aquaria perchlorate concentrations ranged from 0-245 ppb for the control aquaria, 0.073 –0.2 ppm in the 0.1 ppm aquaria, 0.67-0.84 ppm in the 1 ppm aquaria, 6.5-7.7 ppm 10 ppm aquaria, 68-118 in the 100 ppm aquaria, and 743-2,080 in the 1000 ppm aquaria. For the 30 day exposure, aquaria perchlroate concentrations ranged from 11-363

ppb in the control aquaria, 0.078-0.38 in the 0.1 ppm aquaria, 0.6-2 ppm in the 1 ppm aquaria, 6.5-8.2 ppm in the 10 ppm aquaria, 60-75 ppm in the 100 ppm aquaria, and 535-725 ppm in the 1,000 ppm aquaria (See appendix 3.1)

14.4 Test System Observation

Aquaria were observed on a daily basis. The number of individuals that expired each day as well as any abnormal behavior was recorded for each perchlorate concentration. In addition, pH, dissolved oxygen, conductivity, ammonia, and temperature were determined at least 3 times per week.

14.5 Animal Sacrifice and Sample Collections

Mosquitofish were removed from treated aquaria and then rinsed three times in aquaria with reconstituted fresh water. The fish were sacrificed with 1 g/L MS 222 until gill ventilation ceased and the fish did not respond to physical stimuli, according to SOP AQ-1-03 "MS-222 Anesthesia and Euthanasia of Amphibians and Fish". The weight and length of each fish was recorded. The fish were then frozen in liquid nitrogen or preserved in Bouin's fixative (a mixture of 1.5 L picric acid, 0.5L 37% formalin and 0.1L glacial acetic acid) in scintillation vials. Individuals used for perchlorate tissue concentration were wrapped in aluminum foil and frozen by immersion in liquid nitrogen. Perchlorate concentration in tissues was determined by extracting the fish according to SOP AC-2-15 "Extraction and Cleanup of Tissue Samples to be Analyzed for Perchlorate". Animals were pooled to obtain sufficient tissue for analysis (a minimum of approximately 5 g for perchlorate analysis). Fish were processed for histology according to SOP AQ-2-03 "General Histological Processing of Thyroid Follicles in Small Fish". For each fish, ten follicular epithelial cells were measured from each of 10 follicles (100 cells total). Cell height was recorded to the nearest micron. Follicle cell hyperplasia (as evidenced by multiple layers of follicle epithelium, rather than just one layer) was also noted. One fish from each aquaria was dissected and the blood was removed by using a freezing solution. The liver, head, and blood were preserved in liquid nitrogen for future analysis.

Channel catfish were sacrificed with 1.5 g/L MS 222 until gill ventilation ceased and the animal did not respond to physical stimuli, according to SOP AQ-1-03 "MS-222 Anesthesia and Euthanasia of Amphibians and Fish". Fish were then dissected to remove the liver, kidney, gill, gonads, GI tract, head, and fillet. All of the gonads were pooled into one sample because of tissue size. The kidneys, livers, and gills were pooled into four samples per tissue type. The GI tracts, heads, and fillets each composed one sample. Perchlorate in these tissues was extracted according to SOP AC-2-15 "Extraction and Cleanup of Tissue Samples to be Analyzed for

Perchlorate".

For labeling purposes, the letters in parentheses were used: Whole body (WB); fillet (FL); liver (LV); gill (GL); GI tract (GI); gonad (GD); whole blood (BD); head (HD), kidney (KY).

Labeling: samples were labeled with a unique ID number according to the following scheme:

LP (laboratory perchlorate exposure)– sample number- organ (2 letter abbreviation).

E.g., LP-0001-LV is the liver sample from fish # 0001.
LP-0001-FL is the fillet from fish # 0001

The individual fish or tissues that were pooled for determination of perchlorate uptake were listed on form # 249 and reassigned a sample number (LP-BB-2D-01: the first sample for body burden analysis from the 2 day laboratory exposure).

Information included on the label was project number, sample ID, date collected, exposure time period, exposure concentration, and protocol number (SOP IN-03-02 Sample Labeling/Logging Procedure). Samples were also labeled with TIEHH/TTU. All of this information was recorded on the fish dissection/tissue collection sheet. Fish weight and standard length was also recorded on the fish dissection/tissue collection sheet.

14.6 Endpoint Analysis

Perchlorate concentration in tissues was extracted according to SOP AC-2-15 "Extraction and Cleanup of Tissue Samples to be Analyzed for Perchlorate". Analysis and quantification of perchlorate in aquarium water or extracted from tissues was according to SOP AC-2-11 "Analysis of Perchlorate by IC". Thyroid histology was analyzed according to SOP AQ-2-03 "General Histological Processing of Thyroid Follicles in Small Fish".

15. STATISTICAL METHODS

Data from the thyroid histology and from the mosquitofish and catfish tissue perchlorate uptake were analyzed by two-way analysis of variance.

16. PROTOCOL CHANGES/REVISIONS:

See attached change in study documentation forms.

17. RESULTS

The project milestones and deliverables stated in the original proposal were development

of an exposure protocol and generation of preliminary data. The development of the protocol has been accomplished. Recurrent equipment failure prevented radioimmunoassasy analysis of whole body thyroid hormone concentrations. Thus, rather than request additional extensions and further delay completion of the project, it was decided to focus more effort on thyroid histology instead. Radioimmunoassays would have provided only marginally more information than histology provided. Thus, the money and effort allocated to do thyroid hormone analysis was redirected to additional thyroid histological analysis in order to provide the information necessary for meeting deliverables (i.e., effects of perchlorate on thyroid function in fish). Histology analysis was originally proposed to provide preliminary data, as preliminary data was part of the milestones, but instead it was possible to analyze all of the samples and obtain final data for the thyroid histology.

Because of this, another assay, examination of thyroid histology has been initiated. Regardless of the time of exposure, our data indicated that perchlorate induces an increase of the epithelial cell height (Table 1). Two-way ANOVA indicated there is a difference between epithelial cell heights depending upon the time of exposure and upon the concentration of the dose. In reference to time, there was no difference between the 2 and 10 day exposure, but these two were both different than the 30 day exposure. In reference to the concentration of the dose, there was a difference between the fish exposed to 10, 100, and 1,000 ppm compared to the control group. Follicular epithelial hyperplasia was observed in almost all fish sampled after the 30 day exposure to sodium perchlorate.

Regardless of the exposure time, the concentration of perchlorate in body burden of mosquitofish was not detectable when fish were exposed to a concentration at or below 1 ppm. At higher doses (10 to 1000 ppm), the body burden concentration of perchlorate was almost 10 x less than seen in the water. It does not appear that perchlorate bioaccumulates in fish tissues (Table 2). Two-way ANOVA indicated that there was no difference in perchlorate uptake depending upon the time period, but there was a difference depending upon the concentration of the dose. Uptake of perchlorate in fish exposed to 1,000 ppm sodium perchlorate was largely different than exposure to all other concentrations, and only marginally different in fish exposed to 100 ppm compared to lower concentration.

The tissue concentrations for catfish show that the head had the greatest concentration of perchlorate, followed by the fillet, then the GI tract and kidney. The largest concentration of perchlorate, in the head, was still almost 4 x less than that seen in the water (Table 3).

Table 1. Mean (± SD) thyroid follicle epithelial cell height (microns) of adult, female mosquitofish exposed to perchlorate for 2, 10 and 30 days.

Doses (ppm)	Exposure		
	2 days	10 days	30 days
0	4.32 ± 0.90	3.98 ± 0.68	4.22 ± 0.80
0.1	4.59 ± 0.46	3.81 ± 0.47	4.64 ± 0.75
1	5.03 ± 1.20	4.39 ± 1.28	5.68 ± 1.69
10	4.70 ± 1.13	4.52 ± 1.10	6.95 ± 1.59
100	5.04 ± 0.82	5.56 ± 2.52	6.89 ± 3.37
1000	4.58 ± 0.75	4.75 ± 1.18	6.79 ± 1.11

Table 2. Concentrations of perchlorate (ppb) in adult, female mosquitofish exposed to perchlorate in water for 2, 10 and 30 days.

[a]Perchlorate was detected, but the concentration was not high enough to accurately quantify.
[b]Perchlorate detected in only 1 sample of 5 analyzed

Doses (ppm)	Time of exposure		
	2 days	10 days	30 days
0	Trace[a]	Trace[a]	Trace[a]
0.1	Trace[a]	Trace[a]	Trace[a]
1	Trace[a]	204.8 ± 458.0[b]	50.0 ± 111.8[b]
10	2612.4 ± 2481.4	749.6 ± 1039.3	1245.2 ± 618.7
100	16031.0 ± 6389.7	10094.8 ± 1268.0	9722.2 ± 1055.6
1000	77382.2 ± 28267.7	69941.2 ± 21046.9	85404.8 ± 20228.8

Table 3. Concentrations of perchlorate in channel catfish tissues from fish exposed to perchlorate in water.

[a]Perchlorate was detected, but the concentration was not high enough to accurately quantify.

Tissue Type	Concentration (ppb)	Sample Size
Kidney	950.25	4
Liver	168	4
Gill	478	4
Gonad	Trace[a]	1
GI Tract	1318.25	16
Head	25422.13	16
Fillet	7260.75	16

18. DISCUSSION

The protocol outlined above has been adopted in order to determine effects of perchlorate-exposed fish in the laboratory. Further analysis of the samples may suggest additional refinements to the protocol.

Throughout the mosquitofish experiments, death and sickly appearance (lethargic, visible symptoms of disease) were more common in the three highest concentrations, but also appeared in the controls and lower concentrations. Some of the fish exposed to the highest concentration of perchlorate exhibited erratic swimming, bent backs, and discoloration in the back to tail areas. During the catfish exposure, four fish died. The fish appeared lethargic throughout the experiment, but no other abnormal behavior was observed.

Perchlorate anion seems to accumulate mostly in the head, however, the high concentrations of perchlorate in the GI tract may have been due to intake along with food or incidental drinking of the water during feeding. The next highest concentrations were in the kidney and then the gill. If the highest sensitivity for monitoring uptake of perchlorate is desired, then analysis should focus on these tissues.

19. STUDY RECORDS AND ARCHIVE:

Study Records will be maintained at The Institute of Environmental and Human Health (TIEHH) Archive for a minimum of one year after study completion date.

20. REFERENCES:

Manzon RG and Youson JH. 1997. Immunocytochemical and morphometric study of TSH, PRL, GH, and ACTH cells in Bufo arenarum larvae with inhibited thyroid function. Gen. Comp. Endocrinol. 98: 166-176.

Miranda, LA, Paz, DA, Dezi, RE and Pisano, A. 1996. Immunocytochemical and morphometric study of TSH, PRL, GH, and ACTH cells in Bufo arenarum larvae with inhibited thyroid function. Gen. Comp. Endocrinol. 98: 166-176.

21. APPENDICES:

Study Protocol
Changes to Study and Notes to File Documentation

Appendices

1. Study Protocol
2. Changes to Study and Notes to File Documentation
   - 2.1 Change in Study 1
   - 2.2 Change in Study 2
   - 2.3 Change in Study 3
   - 2.4 Change in Study 4

Project No. T9700.11

A STUDY PROTOCOL

ENTITLED

Uptake of Ammonium Perchlorate and Thyroid Status in Native Fish

STUDY NUMBER: FISH-01-1

SPONSOR:
United States Air Force
IERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

ADMINISTRATOR: The Institute of Environmental and Human Health

TESTING FACILITY

Name/Address:

The Institute of Environmental and Human Health
Texas Tech University/Texas Tech University Health Sciences Center
PO Box 41163
Lubbock, Texas 79409-41163

Test Facility Management: Dr. Ronald Kendall

Study Director: Dr. Christopher Theodorakis

PROPOSED EXPERIMENTAL
START DATE: February 9, 2001

1. **DESCRIPTIVE STUDY TITLE:** Uptake of Ammonium Perchlorate and Thyroid Status in Native Fish

2. **STUDY NUMBER:** FISH-01-1

3. **SPONSOR:** United States Air Force
IERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

4. **TESTING FACILITY NAME & ADDRESS:**
The Institute of Environmental and Human Health
Texas Tech University
PO Box 41163
Lubbock, Texas 79409-41163

5. **PROPOSED EXPERIMENTAL START & TERMINATION DATES:**
Start Date: (date of chemical application) February 9, 2001

Termination Date: (date of last data collected) September 31, 2001

6. **KEY PERSONNEL:**
Christopher Theodorakis, Study Director
Ronald Kendall, Testing Facility Management
Todd Anderson, Analytical Chemist
Ryan Bounds, Quality Assurance Manager

7. **DATED SIGNATURES:**

Ronald Kendall for Christopher Theodorakis — Dr. Christopher Theodorakis, Study Director

Ronald Kendall — 2/13/01 — Dr. Ronald Kendall, Testing Facility Management

2/13/01 — Mr. Ryan Bounds, Quality Assurance Manager

Todd Anderson — 2-13-01 — Dr. Todd Anderson, Analytical Chemist

8. **REGULATORY COMPLIANCE STATEMENT:**

Quality Control and Quality Assurance

This study will be conducted in accordance with established Quality Assurance program guidelines and in compliance, where appropriate and possible, with Good Laboratory Practice Standards (40 CFR Part 160, August 17, 1989).

Document Control Statement

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The Institute of Environmental and Human Health
Texas Tech University
PO Box 41163
Lubbock, Texas 79409-211163

9. **STUDY OBJECTIVES / PURPOSE:**

To determine kinetics of uptake and relative tissue distributions of ammonium perchlorate in native fish species.

10. **TEST MATERIALS:**

Test Chemical name: Ammonium perchlorate
CAS number: 7790-98-9
Characterization: determination of strength, purity, stability, homogeneity, etc
Source: Aldrich Chemical Company

Reference Chemical name:
ultrapure water with added sea salts ("Instant Ocean®" or any other brand of sea salts with identical or nearly identical composition).
CAS Number: Not applicable
Characterization: determination of strength, purity, stability, homogeneity, etc
Source: City tap water that has been run through reverse osmosis and a de-ionizer to convert it to ultrapure water. 60 mg/L salts will be added.

11. **JUSTIFICATION OF TEST SYSTEM:**

Ionic perchlorate alters thyroid homeostasis in fishes and amphibians as well as other vertebrates (Miranda et al., 1996; Manzon and Youson, 1997). Because of the important role played by hormones in animal development and reproduction, endocrine disruption is

likely to lead to serious impairments in growth, reproductive fitness, and consequently, fish and wildlife population stability as well as human health.

12. **TEST ANIMALS** (Where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test system):

Species: *Gamgusia affinis,* Western mosquitofish; *Amerius spp.* bullhead catfish (any other species native to Texas weighing at least 100 g may be substituted for bullhead catfish).

Strain: Feral organisms or bred in hatcheries

Age: Adults.

Number: Approximately 2700 mosquitofish, and 120 catfish

Source: Captured in the wild, or purchased from hatcheries, Carolina Biological Supply or other commercial suppliers

13. **PROCEDURE FOR IDENTIFYING THE TEST SYSTEM:**
The test system will consist of laboratory exposures constructed according to the experimental design described below. Wild fish will be identified in the field (upon capture) by the project manager or personel trained in the identification of such fish. Identity of all fish will be confirmed in the laboratory by visual inspection before tests are begun. Aquaria will be labeled with the project number, test system, date of collection, concentration, and person responsible.

14. **EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL:**
Mosquitofish will be exposed to five concentrations, and catfish will be exposed to three concentrations, of ammonium perchlorate plus zero concentration or control. Fish will be placed into precleaned aquaria. Aquaria will be cleaned by washing each aquarium according to SOP AQ-1-02 "Cleaning Glassware and Aquaria for Perchlorate Assays". For exposures, aquaria will be located on shelves capable of supporting such weight. Each shelf will hold four 80L aquaria (for the catfish) or six 20L aquaria (for the mosquitofish). The experimental design will consist of a randomized block design, with each shelf constituting a block. The arrangement of the aquaria within each block will be randomized in order to avoid effects due to gradients in light, temperature, volatile chemicals in the laboratory, etc. Determination of the arrangement of the aquaria or beakers within each block by a random number generator, random number table or by rolling dice. Each block will contain at least 1 beaker or aquarium of each treatment.

Fish will be placed in the aquaria in random order, within blocks, using the procedure described below.

For example, for the mosquitofish, each block will consist of 6 aquaria, each with 15 fish. There will be 5 blocks, for a total of 450 fish. All fish will be placed in one or more buckets. Within each block, each aquarium will be assigned a number from 1-6. A random number generator will be used to randomly order the numbers 1-6, and this will be done 15 times: e.g., 341256, 465123, 564312, 435126, 345126, 163452, 234516, 132465, 235461, 213465, 623415, 342156, 623415, 614325, 534261. A pair of dice, a random number table, or a computerized random number generator can be used for this purpose. The fish will be placed into the 6 aquaria in the 1st block according to this list of numbers. For example, one fish will be placed into aquarium 3, then aquarium 4, aquarium 1, aquarium 2, aquarium 5 and finally aquarium 6. A second fish will be placed in each aquarium in the order 465123. A third fish will then be placed in each aquarium in the order 564312, and so on, until there are 15 fish in each aquarium. This will then be done for the other 4 blocks in the experiment. A similar procedure will be carried out for the catfish, except that there will be 4 aquaria per block and 1 fish per aquarium.

For both species, only females will be used in order to avoid sex effects. Mosquitofish that weigh approximately 0.7-1.2 g, and catfish that weigh approximately 120-150 g, will be used for these experiments.

15. METHODS:

15.1 Test System acquisition, quarantine, acclimation

Fish will be obtained from the wild populations, commercial vendors, or fish hatcheries. If fish are captured in the wild, they will be transported back from the field in plastic buckets or other containers with constant aeration. Upon arrival to the lab, they will be treated commercially available antibiotics for 5 days, as instructed by the manufacturer. After five days, any debris at the bottom of the tank and 1/3 of the tank water will be removed using a siphon hose or electric pump and replaced with fresh water. Fresh water will consist of reverse osmosis (RO) water supplemented with 60 mg/L Instant Ocean sea salts or other brands of identical composition. Fresh water will be replaced in each tank by siphon or electric pump from a reservoir (e.g., 100 gallon aquarium). Each day, debris at the bottom of the tank will be cleaned by suction. Water will be continuously aerated and filtered using mechanical and biological filtration. Animal husbandry will be according to SOP AQ-1-08, "General Fish Husbandry" and SOP AQ-1-09 "Mosquitofish *Gambusia spp.* Husbandry". Total acclimatization period will be a minimum of one week. Once acclimated, fish will be exposed to ammonium perchlorate dissolved in water. Mosquitofish will be fed commercial flake goldfish food at the rate of 5 mg per gram of fish, on a daily basis. Catfish will be fed pelleted food at the same rate. Debris and uneaten food will be removed from the bottom of the tank 1-3 hours post feeding.

15.2 **Test Condition Establishment**

Exposures will begin after fish have become acclimatized.

15.3 **Test Material Application**

Fish will be placed into beakers or aquaria containing various concentrations of ammonium perchlorate, with 15 fish per aquarium and 5 replicate aquaria per treatment. Stock solutions of 1 g/L, 10 g/L, and 100 g/L perchlorate in reconstituted fresh water will be used to dose the fish. The mosquitofish tanks will be filled with 15 L water, and the catfish tanks will be filled with 60L of water, and an appropriate amount of stock solution will be added according to the desired concentration of the aquarium water. Every other day, debris will be cleaned out of the aquaria and 1/3 of the water will be replaced in each tank with undosed water (as described in 15.1), and perchlorate stock solution will be added to maintain the desired concentration.

Rates/concentrations: Fish will be exposed to 0, 0.1, 1.0, 10, 100, and 1000 ppm (mosquitofish or alternative small species) or 0, 10, 100 and 1000 ppm (catfish or alternative large species) perchlorate in water.

Frequency: Five replicate tanks of each concentration will be continually exposed for 1, 2, 5, 10, 20 or 30 days (i.e., 6 concentration x 6 exposure periods = 36 treatments in all for mosquitofish, 4 concentrations x 6 exposure periods = 24 treatments in all for catfish). Exposure periods may be extended if initial results warrant.

Route/Method of Application: Route will be via dermal, oral and respiratory exposure as the chemical will be in the beaker/aquaria water.

Stock solutions for study will be mixed in precleaned glass containers as indicated in SOP AQ-1-02-01. Stock solutions will be made by dissolving ammonium perchlorate in reconstituted fresh water (60 mg/L Instant Ocean® sea salts or equivalent, in ultrapure water, pH adjusted to 7.4 with 1N HCl or 1N NaOH, as appropriate). The appropriate amount of ammonium perchlorate compound will be weighed on a calibrated balance, and mixed into reconstituted fresh water. The pH will be checked on a calibrated pH meter (calibrate according to SOP IN-4-06) and adjusted, if necessary, as above. After 1/3 of the aquarium water has been removed and replaced (see above instructions), an appropriate amount of stock solution will be added to adjust the concentration to the original value.

For the mosquitofish, the 1 g/L stock will be used for the 0.1 ppm (0.1 mg/L) treatments; the 10 g/L stock will be used for the 1 ppm (1 mg/L) and 10 ppm (10 mg/L), treatments;

and the 100 g/L stock will be used for the 100 and 1000 ppm (100 and 1000 mg/L) treatments. For example, if there is 15L water in a mosquitofish tank, 5L will be removed and replaced every other day. For the aquaria that have the 1 ppm and 10 ppm, 0.005 L (5 ml) of perchlorate stock solution will be added to the 1 ppm aquarium and 0.05 L (50 ml) will be added to the 10 ppm aquarium (e.g., (5L x 10 mg/L)/10,000 mg/L = 0.005 L). Five ml of stock will be measured out in a 5 ml pipette; 50 ml of stock will be measured out in a 50 ml pipette.

For the catfish, the 200 g/L stock will be used for all treatments. For these fish, 20L of water will be removed every other day. Thus, for the 10 ppm treatment, 1 ml of stock will be added to the tanks after water change, for the 100 ppm, 10 ml will be added, etc. For adding 1 ml, 10 ml, and 100 ml of stock to an aquarium, a 1 ml, 10 ml or 50 ml pipette will be used, respectively

Justification for Exposure Route: Exposure by environmental waters is most appropriate because fish respire, ingest, and are dermally exposed to chemicals in the waters in which they live.

Exposure Verification: A sample of each concentration of treated water will be collected at least every 3rd day and whenever animals are removed from the aquarium for analysis. The concentration of perchlorate in the water will be tested using ion chromatography.

15.4 **Test System Observation**

Tanks or beakers will be observed on a daily basis. The number of individuals that expire each day will be recorded for each perchlorate concentration. In addition, pH, dissolved oxygen, conductivity, temperature, and any other water chemistry parameters deemed appropriate by the project manager will be determined at least 3 times per week.

15.5 **Animal Sacrifice and Sample Collections**

Mosquitofish will be weighed, sacrificed with an overdose of MS 222, and either frozen in liquid nitrogen or preserved in either Bouin's fixative (a mixture of 1.5 L picric acid, 0.5L 37% formalin and 0.1L glacial acetic acid) or 10% buffered formalin (at least 10 ml per gram of tissue for each) in scintillation vials or equivalent glass vials. An overdose of MS222 will consist of immersing the animal in 1.5 g/L MS222 for at least 60 seconds after all gill ventilation has ceased, according to SOP AQ-1-03 "MS-222 Anesthesia and Euthanasia of Amphibians and Fish". Individuals used for perchlorate tissue concentration or whole body thyroid hormone analysis will be wrapped in aluminum foil or placed in cryogenic tubes suitable for liquid-phase liquid nitrogen and will be frozen by immersion in liquid nitrogen. Perchlorate concentration in tissues will be extracted according to SOP AC-2-15 "Extraction and Cleanup of Tissue Samples to be Analyzed for Perchlorate". Whole body thyroid hormones will be extracted and purified according

to SOP MT-2-08 "Extraction of Thyroid Hormones From Animal Tissues" and MT-2-09 "Ion Exchange Purification of Tissue Extracts for Thyroid Hormone Radioimmunoassay". Animals may be pooled to obtain sufficient tissue for analysis (a minimum of approximately 5 g for perchlorate analysis and 2 g for thyroid hormone). Fish will be processed for histology according to SOP AQ-2-03 "General Histological Processing of thyroid Follicles in Small Fish". If possible, blood may be collected by anesthetizing the animal as described below, severing the caudal peduncle near the anal fin, and blood will be collected using heparinized or EDTA-treated microhematocrit tubes according to SOP AQ-3-06 "Collection Of Blood From Fish". Fish may be pooled to collect at least 50 µl blood. Blood will then be centrifuged as described below.

Bullhead catfish (or other surrogate) will be anesthetized in 0.5 g/L MS222 until the animal loses righting reflex and does not respond to physical stimuli, but before gill ventilation ceases, according to SOP AQ-1-03 "MS-222 Anesthesia and Euthanasia of Amphibians and Fish". Blood will then be collected using 3 ml, EDTA-treated Becton-Dickinson vaccutainer tubes or 5 ml syringes treated with 0.5 m EDTA according to SOP AQ-3-06, "Collection Of Blood From Fish". Blood will be transferred to microcentrifuge tubes and spun within 5 minutes of collection in a microcentrifuge at 3000-5000 rpm for 3-5 min. Plasma will then be decanted with a Pasteur pipette or micropipette into a cryogenic tube suitable for liquid-phase liquid nitrogen and frozen by immersion in liquid nitrogen. Fish will then be sacrificed by cervical scission and dissected to remove any combination (or all) of the following organs (for labeling purposes, the letter in parentheses will be used, see below):

Whole body (WB); fillet (FL); liver (LV); gill (GL); GI tract (GI); gonad (GD); whole blood (BD); plasma (PS); head (HD).

For tissue distribution of perchlorate in catfish or surrogate species, fillet, liver, gill, GI tract, and head will be collected. Perchlorate in these tissues will be extracted according to SOP AC-2-15 "Extraction and Cleanup of Tissue Samples to be Analyzed for Perchlorate". If the gonads are not atrophied, they will also be collected. If at least 1 ml of whole blood can be collected, it will also be analyzed for perchlorate analysis. For mosquitofish, whole bodies will be used.

Labeling: samples will be labeled with a unique ID number according to the following scheme:

LP (laboratory perchlorate exposure)– sample number- organ (2 letter abbreviation).

E.g., LP-0001-LV is the liver sample from fish # 0001.
LP-0001-FL is the fillet from fish # 0001

If samples are to be divided into subsamples, then a suffix is attached. E.g., if the liver sample above is divided into 3 subsamples, these subsamples will be labeled:

LP-0001-LV.1
LP-0001-LV.2
LP-0001-LV.3

If samples are to be composited, then the prefix will be LPC. (e.g., LPC-0001-WB is composite # 1, consisting of whole bodies). The number of individual samples comprising the composite should be indicated on the fish dissection/tissue collection form and/or in a bound laboratory notebook.

Minimum information to be included on the label is project number and unique ID (SOP IN-03-02 Sample Labeling/Logging Procedure). Additional information can include species, date collected and sex (if known), in decreasing order of importance. All of this information should be recorded on the fish dissection/tissue collection sheet and/or bound laboratory notebook. Fish weight should also be recorded, and standard length (from the tip of the nose to the end of the caudal peduncle) may also be included on the form on in the notebook. Any information not determined should be entered as "ND".

15.6 **Endpoint Analysis**

Perchlorate concentration in tissues will be extracted according to SOP AC-2-15 "Extraction and Cleanup of Tissue Samples to be Analyzed for Perchlorate". Analysis and quantification of perchlorate in aquarium water or extracted from tissues will be according to SOP AC-2-11 "Analysis of Perchlorate by IC". Thyroid hormones in plasma or extracted from tissues will be analyzed according to MT-2-10 "Radioimmunoassay for Thyroid Hormones". Thyroid histology will be analyzed according to SOP AQ-2-03 "General Histological Processing of thyroid Follicles in Small Fish".

16. PROPOSED STATISTICAL METHODS:

To statistically determine the differences between treatments in terms of histological endpoints, thyroid hormone or perchlorate body concentrations, 2-way ANOVA will be used to determine effects of concentration and time of exposure. Correlation and regression analysis may also be used to determine the relationship between response (body burden, thyroid function) vs. dose or vs. time.

17. REPORT CONTENT/RECORDS TO BE MAINTAINED:

Records to be maintained include: Room temperature and water temperature, dissolved oxygen, salinity, and pH will be collected. Date, time, and amount of feedings per tank will be recorded. Relative tissue distribution in bullhead catfish, relationship between

perchlorate body burden and exposure concentration, and preliminary data on thyroid function and/or histology will be included in the report.
Report content will include presentation of data, interpretation, and discussion of the following endpoints:

List individual endpoints and analyses.
Interpretation of all data, including statistical results
Discussion of the relevance of findings
List of all SOPs used
List of all personnel

18. **RECORDS TO BE MAINTAINED / LOCATION:**
The final report will be delivered to the Sponsor on or before November 14, 2001. Copies of all data, documentation, records, protocol information, as well as the specimens shall be sent to the Sponsor, or designated delivery point, upon request. All data, the protocol and a copy of the final report shall be archived at the testing facility.

19. **QUALITY ASSURANCE:**
The Quality Assurance Unit will inspect the study at intervals to insure the integrity of the study. Written records will be maintained indicating but not limited to the following: date of inspection, study inspected, phase inspected, person conducting the inspection, findings and problems, recommended and taken action, and any scheduled re-inspections. Any problems likely to effect study integrity shall be brought to the immediate attention of the Study Director. The Quality Assurance Unit will periodically submit written status reports on the study to management and the Study Director.

20. **PROTOCOL CHANGES / REVISIONS:**
All changes and/or revisions to the protocol, and the reasons therefore, shall be documented, signed and dated by the Study Director and Test Facility Manager and maintained with the protocol and the Quality Assurance Unit.

21. **REFERENCES:**

Manzon RG and Youson JH. 1997. Immunocytochemical and morphometric study of TSH, PRL, GH, and ACTH cells in Bufo arenarum larvae with inhibited thyroid function. Gen. Comp. Endocrinol. 98: 166-176.

Miranda, LA, Paz, DA, Dezi, RE and Pisano, A. 1996. Immunocytochemical and morphometric study of TSH, PRL, GH, and ACTH cells in Bufo arenarum larvae with inhibited thyroid function. Gen. Comp. Endocrinol. 98: 166-176.

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Form No. 014 Rev. 3.06/00
Project No.: T9700.11
*Change No: 1
Page: 1 of 1

Change In Study Documentation Form

The following documents changes in the above referenced study:

Check One: X Amendment ____Deviation ____ Addendums

Document Reference Information
Check One: X Protocol ____ SOP ____ Other ____
Title: Uptake of Ammonium Perchlorate and Thyroid Status in Native Fish
Dated: February 13, 2001
Document # (if appropriate): FISH 01-1
Page #(s): 1, 2, 3, 4, 5, and 6
Section #: Title, 1, 9, 10, 14, 15.1, and 15.3
Text to reference: Title - ... Ammonium Perchlorate ...; Section 1 - ... Ammonium Perchlorate ...; Section 9 - ... ammonium perchlorate ...; Section 10 - ... Ammonium perchlorate ... CAS number: 7790-98-9 ... Source: Aldrich Chemical Company; Section 14 - ... ammonium perchlorate ..., Section 15.1 - ... ammonium perchlorate ...; Section 15.3 - ... ammonium perchlorate ... ammonium perchlorate ... ammonium perchlorate ...

Change in Document: Title - ... Sodium Perchlorate ...; Section 1 - ... Sodium Perchlorate ...; Section 9 - ... sodium perchlorate ...; Section 10 - ...Sodium perchlorate ... CAS number: 7601-89-0 ... Source: EM Science; Section 14 - ... sodium perchlorate ...; Section 15.1 - ...sodium perchlorate ...; Section 15.3 - ... sodium perchlorate ... sodium perchlorate ... sodium perchlorate ...

Justification and Impact on Study: Since forms of Ammonium are toxic to fish, sodium perchlorate was used instead of ammonium perchlorate to avoid complications with working with ammonium. This study is interested in effects of the perchlorate ion so this change will not affect the study.

Submitted by: Signature: Carrie Bradford Date: 9/20/01

Authorized by: Study Director: Clin Hunter Date: 9/27/01

Received by: Quality Assurance Unit: Brian Buhrell Date: 3/28/02

* Sequentially numbered in order of the date that the change is effective

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Document Reference Information
Check One: X Protocol ____ SOP ____ Other _____
Title: Uptake of Ammonium Perchlorate and Thyroid Status in Native Fish
Dated: February 13, 2001
Document # (if appropriate): FISH 01-1
Page #(s): 4, 5, 6, 7, 8 and 9
Section #: 12, 15.1, 15.3, 15.5, 15.6, and 17
Text to reference: Section 12 - … *Gambusia affinis*, Western Mosquitofish… approximately 2700 mosquitofish; Section 15.1 - Each day, debris at the bottom of the tank will be cleaned by suction … Debris and uneaten food will be removed from the bottom of the tank 1-3 hours post feeding; Section 15.3 – Stock solutions of 1 g/L, 10 g/L, and 100 g/L perchlorate in reconstituted fresh water will be used to dose the fish … Five replicate tanks of each concentration will be continually exposed for 1, 2, 5, 10, 20, or 30 days (i.e., 6 concentrations x 6 exposure periods = 36 treatments … perchlorate in reconstituted fresh water … pH adjusted to 7.4 with 1N HCl or 1N NaOH … mixed into reconstituted fresh water … For the aquaria that have the 1 ppm and 10 ppm, 0.005 L (5mL) of perchlorate stock solution will be added to the 1 ppm aquarium and 0.05 L (50 mL) will be added to the 10 ppm aquarium (e.g., (5L x 10 mg/L)/10,000 mg/L = 0.005 L). Five mL of stock will be measured out in a 5 mL pipette; 50 mL of stock will be measured out in a 50 mL pipette … A sample of each concentration of treated water will be collected at least every 3rd day and whenever animals are removed from the aquarium for analysis; Section 15.5 - … immersing the animal in 1.5 g/L MS222 … Whole body thyroid hormones will be extracted and purified according to SOP MT-2-08 "Extraction of Thyroid Hormones From Animal Tissues" and MT-2-09 "Ion Exchange Purification of Tissue Extracts for Thyroid Hormone Radioimmunoassay" …blood will be collected using heparinized or EDTA-treated microhematocrit tubes according to SOP AQ-3-06 …; Section 15.6 – Thyroid hormones in plasma or extracted from tissues will be analyzed according to MT –2-10 "Radioimmunoassay for Thyroid Hormones."; Section 17 – Records to be maintained include: Room temperature and water temperature … Date, time, and amount of feedings per tank will be recorded.

Change in Document: Section 12 - … *Gambusia holbrooki*, Eastern Mosquitofish… approximately 1800 mosquitofish; Section 15.1 - Every other day, debris at the bottom of

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the tank will be cleaned by suction ... This sentence should be deleted.; Section 15.3 – Stock solutions of 1 g/L, 10 g/L, and 100 g/L perchlorate in ultrapure water will be used to dose the fish ... Five replicate tanks of each concentration will be continually exposed for 1, 2, 10, and 30 days (i.e., 6 concentrations x 4 exposure periods = 24 treatments ... perchlorate in ultrapure water ... pH adjusted to 7.4 with 0.1N HCl, 1N HCl, 0.1N NaOH, and 1N NaOH ... mixed into ultrapure water ... For the aquaria that have the 1 ppm and 10 ppm, 0.0005 L (0.5 mL) of perchlorate stock solution will be added to the 1 ppm aquarium and 0.005 L (5 mL) will be added to the 10 ppm aquarium (e.g., (5L x 10 mg/L)/10,000 mg/L = 0.005 L). Five mL of stock will be measured out in a 5 mL pipette; 0.5 mL of stock will be measured out in a 0.5 mL pipette ... A water sample from each aquarium will be taken at the beginning of the exposure period, at least once a week throughout the exposure period, and at the end of the exposure period; Section 15.5 - ... immersing the animal in 1.0 g/L MS222 ... Whole body thyroid hormones will be extracted and purified according to DBS SOP IN-2-01-01 "Extraction of Thyroid Hormones From Animal Tissues" and DBS SOP IN-2-05-01 "Ion Exchange Purification of Tissue Extracts for Thyroid Hormone Radioimmunoassay" ... blood will be collected by freezing intact red blood cells/nuclei according to SOP AQ-3-06 ...; Section 15.6 – Thyroid hormones in plasma or extracted from tissues will be analyzed according to DBS SOP IN-2-04-01 "Radioimmunoassay for Thyroid Hormones."; Section 17 – Records to be maintained include: Water temperature ... Date and time of feedings for the aquaria will be recorded.

Justification and Impact on Study: Section 12 - *Gambusia holbrooki* was used in place of *Gambusia affinis* because of availability of species. These species are very closely related and so this will have no effect on the study. Less fish were used since not all the exposures were completed; Section 15.1 - Section 15.3 discusses the proper method of cleaning the tanks ... Since aquaria receive a 1/3 water change every other day, not much debris accumulated on the bottom of the aquaria so daily cleaning was not necessary.; Section 15.3 – Ultrapure water was used to make stock solutions instead of reconstituted fresh water ... Only these four exposure periods were completed because of time constraints ... Ultrapure water was used to make stock solutions instead of reconstituted fresh water ... Because of large changes in the pH when using 1N HCl and 1N NaOH, lower concentrations of these were used ... Ultrapure water was used to make stock solutions instead of reconstituted fresh water ... 10 mg/L is equivalent to 10 ppm so the example in parentheses should be for the 10 ppm aquaria not the 1 ppm aquaria ... Because of the time and expense involved in analyzing water samples, less samples were collected; Section 15.5 - 1.5 g/L MS222 solution was too strong for small fish and so 1.0 g/L solution was used instead ...Since whole body thyroid hormone analysis will be conducted in the Biology Department, using the Biology Department's equipment, these SOP's were not converted to the TIEHH format ... because the fish are so small, an

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alternative method of blood collection was used.; Section 15.6 – Since thyroid hormone analysis will be conducted in the Biology Department, using the Biology Department's equipment, these SOP's were not converted to the TIEHH format.;Section 17 – Room temperature and amount of feeding per tank were not recorded.

Submitted by: Signature: Carri Bradford Date: 9/20/01

Authorized by: Study Director: John Henderson Date: 9/27/01

Received by: Quality Assurance Unit: Brian Bridwell Date: 3/28/02

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The following documents changes in the above referenced study:

Check One: ____ Amendment ____ Deviation _X_ Addendums

Document Reference Information
Check One: _X_ Protocol ____ SOP ____ Other _____
Title: Uptake of Ammonium Perchlorate and Thyroid Status in Native Fish
Dated: February 13, 2001
Document # (if appropriate): FISH 01-1
Page #(s): 7
Section #: 15.5
Text to reference: _____

Change in Document: Section 15.5 – Prior to animal sacrifice, fish will be rinsed three times with reconstituted fresh water to remove perchlorate on the exterior of the body. Fish will be removed from the treatment aquarium and put into the first rinse tank, then they will be transferred to the second rinse tank, then they will be transferred to the last rinse tank. Animals will then be immersed in an overdose of MS-222 and then rinsed once with DI water. Animals will then be measured and weighed. … The abdomen of the fish will be cut open or the fish will be cut in half prior to fixation in Bouin's fixative to allow the Bouin's to get into all the tissues. … Additional organ abbreviation : Kidney (KY)

Justification and Impact on Study: Section 15.5 – Since some of the fish will be used to determine perchlorate uptake into the tissues, it is important that there is no residual perchlorate on the exterior of the body. … It is necessary for the Bouin's fixative to get to all the tissues to assure proper fixation. … The kidney may also be collect for tissue distribution of perchlorate.

Submitted by: Signature: Carri Bradford Date: 9/20/01

Authorized by: Study Director: Clin R Theodorakis Date: 9/27/01

Received by: Quality Assurance Unit: Brian Birdwell Date: 3/28/02

* Sequentially numbered in order of the date that the change is effective

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Document Reference Information
Check One: X Protocol ____ SOP ____ Other _____
Title: Uptake of Ammonium Perchlorate and Thyroid Status in Native Fish
Dated: February 13, 2001
Document # (if appropriate): FISH 01-1
Page #(s): 4, 5, 6, 7, 8
Section #: 12, 14, 15.3, 15.5
Text to reference: Section 12 – *Amerius spp.*, bullhead catfish… 120 catfish; Section 14 – … and catfish will be exposed to three concentrations, …; Each shelf will hold four 80 L aquaria (for the catfish) …; A similar procedure will be carried out for the catfish, except that there will be 4 aquaria per block and 1 fish per aquaria.; Section 15.3 - … catfish tanks will be filled with 60 L of water.; or 0, 10, 100 and 1000 ppm (catfish or alternative large species).; …continually exposed for 1, 2, 5, 10, 20, or 30 days…4 concentrations x 6 exposure periods = 24 treatments in all for catfish.; For the catfish, the 200 g/L stock will be used for all treatments. For these fish, 20 L of water will be removed every other day. Thus, for the 10 ppm treatment, 1 mL of stock will be added to the tanks after water change, for the 100 ppm, 10 mL will be addedd, etc. For adding 1 mL, 10 mL, and 100 mL of stock to an aquarium, a 1 mL, 10 mL, or 50 mL pipette will be used, respectively. Section 15.5 – Bulhead catfish (or other surrogate) will be anesthetized in 0.5 g/L MS222 until the animal loses righting reflex and does not respond to physical stimuli, but before gill ventilation ceases, according to SOP AQ-1-03 "MS-222 Anesthesia and Euthanasia of Amphibians and Fish." Blood will then be collected using 3 mL, EDTA-treated Becton-Dickinson vaccutainer tubes or 5 mL syringes treated with 0.5 m EDTA according to SOP AQ-3-06, "Collection of Blood from Fish." Blood will be transferred to microcentrifuge tubes and spun within 5 minutes of collection in a microcentrifuge at 3000-5000 rpm for 3-5 min. Plasma will then be decanted with a Pasteur pipette or micropipette into a cryogenic tube suitable for liquid-phase liquid nitrogen and frozen by imersion in liquid nitrogen. Fish will then be sacrificed by cervical scission and dissected to remove any combination (or all) of the following organs …

Change in Document: Section 12 – *Ictalurus spp.*, channel catfish… 20 catfrish; Section 14 - … and catfish will be exposed to one concentration (100 ppm), …; Each shelf will hold five 20 L aquaria (for the catfish) …; For the catfish study, each shelf will consist

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of 5 aquaria which will comprise one composite for pooling tissues. Each aquaria will house one fish.; Section 15.3 - … catfish tanks will be filled with 15 L of water.; or 100 ppm (catfish or alternative large species).; Catfish will be continually exposed for 5 days at a concentration of 100 ppm.; For the catfish, the 100 g/L stock will be used for all treatments. For these fish, 5 L of water will be removed every other day. Thus, for the 100 ppm treatment, 5 mL of stock will be added to the tanks after water change by using a 5 mL pipette. Section 15.5 – Channel catfish (or other surrogate species) will be euthanized in 1.5 g/L MS-222 until gill ventilation ceases, according to SOP AQ-1-03 " MS-222 Anesthesia and Euthanasia of Amphibians and Fish." Fish will then be dissected to remove any combination (or all) of the following organs … The organs will be frozen in liquid-phase liquid nitrogen for perchlorate uptake determination.

Justification and Impact on Study: Section 12 – Channel catfish were used instead of bullhead catfish because of availability of species. Since only one experiment was completed, only 20 catfish were used. Section 14 – Because of the difficulty in getting catfish and maintaining them in the lab, only one experiment was completed with 20 replicates of 100 ppm treatment.; Catfish were smaller than originally planned so smaller tanks could be used to reduce the amount of waste water produced.; Since the dosing concentration and time period for the catfish study was the same for all the fish, it was not necessary to set up a randomized block design study.; Section 15.3 – Smaller aquaria were used for the catfish tanks.; only one dosing concentration was used because of difficulties in getting and maintaining catfish in the lab.; only one time period was used because of difficulties in getting and maintaining catfish in the lab.; Since the experiment design was changed, the concentration of stock solution and amount added to each aquaria was also changed. Section 15.5 - All fish were euthanized and dissected to determine the amount of perchlrorate uptake in the different tissues. Blood was not collected from these catfish.

Submitted by: Signature: Carrie Bradford Date: 9/26/01

Authorized by: Study Director: [signature] Date: 9/27/01

Received by: Quality Assurance Unit: Brian Bidwell Date: 3/28/02

* Sequentially numbered in order of the date that the change is effective

A FINAL REPORT

ENTITLED

IN SITU EXPOSURE OF FISH AND AMPHIBIANS FOR DETERMINATION OF CONTAMINANT EFFECTS AT THE LONGHORN ARMY AMMUNITION PLANT, JEFFERSON COUNTY, TEXAS

STUDY NUMBER: FAE-01-01

SPONSOR:
United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

CONTRACT ADMINISTRATOR:
The Institute of Environmental and Human Health
Texas Tech University / TTU Health Sciences Center

Box 41163
Lubbock, Texas 79409-1163

TESTING FACILITY:
The Institute of Environmental and Human Health
Texas Tech University
Box 41163
Lubbock, Texas 79409-1163

TEST SITE:
The Institute of Environmental and Human Health
Texas Tech University
Box 41163
Lubbock, Texas 79409-1163

ANALYTICAL TEST SITE:
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Texas Tech University / TTU Health Sciences Center
Box 41163
Lubbock, Texas 79409-1163

RESEARCH INITIATION: 05/15/2001

RESEARCH COMPLETION: 10/07/2001

Table of Contents

List of Tables and Figures

GOOD LABORATORY PRACTICES STATEMENT

This project, entitled "*IN SITU* EXPOSURE OF FISH AND AMPHIBIANS FOR DETERMINATION OF CONTAMINANT EFFECTS AT THE LONGHORN ARMY AMMUNITION PLANT, JEFFERSON COUNTY, TEXAS", was performed whenever possible in the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989

Submitted By:

Christopher Theodorakis, Ph.D

Date

QUALITY ASSURANCE STATEMENT

This study was conducted under the Institute of Environmental and Human Health's Quality Assurance Program and whenever possible to meet the spirit of the Good Laboratory Practices as outlined in 40 CFR Part 160, August 19, 1989. Any changes in protocol and SOPs were documented in writing and signed by the study director.
The Quality Assurance Officer verbally notified the Study Director of all findings at the time of the inspection. Written audit reports were also submitted to the Study Director and Test Facility Management. Audits were performed for the following phases of the project:

Auditable Research Phase / Activity	Audit Dates		Date written report submitted to Study Director	Date written report submitted to Management
	Start	End		
Final Report and Raw Data Review	2/25/02	3/06/02	3/19/02	3/19/02

Submitted By:

_____ Date _____

Ryan Bounds
Quality Assurance Manager

1. DESCRIPTIVE STUDY TITLE

In Situ Exposure Of Fish And Amphibians For Determination Of Contaminant Effects At The Longhorn Army Ammunition Plant, Jefferson County, Texas.

2. STUDY NUMBER

FAE-01-01

3. SPONSOR

United States Air Force
AFIERA/RSE
2513 Kennedy Circle
Brooks Air Force Base, Texas 78235-5123

4. TESTING FACILITY NAME AND ADDRESS

The Institute of Environmental and Human Health
Texas Tech University
Box 41163
Lubbock, Texas 79409-1163

5. PROPOSED EXPERIMENTAL START & TERMINATION DATES

Start Date: 05/15/2001
Termination Date: 09/31/2001

6. KEY PERSONNEL

Dr. Christopher Theodorakis, Study Director
Dr. Todd Anderson, Analytical Chemist
Dr. Ronald Kendall, Testing Facilities Management/Principal Investigator
Ryan Bounds, Quality Assurance Manager
Emilia Cruz-Li, Technician
Carrie Bradford, Technician

7. STUDY SUMMARY

Western mosquitofish (*Gambusia affinis*) and bullfrog tadpoles (*Rana catesbaena*) were placed in minnow traps in contaminated and reference locations at LHAAP. They were allowed to remain in situ for 1, 5 or 10 days concurrently for fish and for 1, 3 and 5 days for tadpoles. There were 5 cages deployed at each site. After removal, they were weighed, labeled, frozen, and transported back to the laboratory

for perchlorate analysis to evaluate perchlorate uptake from contaminated surface waters.

Mosquitofish were placed in minnow traps (described above) in reference and contaminated sites to evaluate reproductive effects. They were allowed to remain in situ for 6 weeks, or as long as possible. Fish were preserved in 70% ethanol, dissected, and the number of embryos in each female was determined by counting.

Tadpoles were caged in minnow traps and were allowed to remain in the cages for 4-6 weeks, although mortality and predation were high in several sites. After this time, the length of the body, tail and hind limbs were recorded. Developmental effects were assessed by comparing the ratio of tail length/body length or hind limb length/body length between caging sites.

8. STUDY OBJECTIVES / PURPOSE

The objectives for this study were to perform pilot experiments in order to determine feasibility studies and develop assay conditions for in situ exposure for examining effects of perchlorate exposure on metamorphosis of amphibians and reproduction of mosquitofish.

9. TEST MATERIALS

Test Chemical name: Perchlorate anion
CAS number: 7790-98-9
Characterization: Determination of concentration in environmental samples
Source: Wastewater treatment effluent discharge

10. JUSTIFICATION OF TEST SYSTEM

Preliminary surveys of LHAAP have revealed that measurable levels of ammonium perchlorate have been found in surface waters of aquatic systems (i.e., streams, ponds, and bayous) within and adjacent to LHAAP. However, the uptake, bioaccumulation, and tissue distribution of perchlorate in fish and amphibians has not been studied to date. Information on the acute and chronic toxic effects of perchlorate in fish are little known. Previous studies conducted at TIEHH have addressed acute toxicity of perchlorate on zebrafish (*Danio rerio*) and African clawed frogs (*Xenopus laevis*). In addition, studies in scientific literature indicate that thyroid homeostasis is important in reproductive functions such as gonadal development, growth, and embryonic development. Ionic perchlorate alters thyroid homeostasis in fishes and amphibians as well as other vertebrates (Miranda et al., 1996; Manzon and Youson, 1997). By examination of uptake of perchlorate, growth and development of tadpoles and fish fry, and reproduction of fish in cages placed in contaminated water, information can be gained that would be instrumental in extrapolation of laboratory studies to native organisms in the field.

11. TEST ANIMALS (where applicable provide number, body weight range, sex, source of supply, species, strain, substrain, and age of test system)

Species: Western mosquitofish (*Gambusia affinis*) and any amphibian species deemed suitable as determined by abundance of specific aspects of their biology.
Strain: Wild animals.
Age: Various.
Number: Maximum of 900 per species.
Source: Captures from natural waters at LHAAP or commercial sources.

12. PROCEDURE FOR IDENTIFYING THE TEST SYSTEM

The test system was natural waters within LHAAP. TTU and private contractors have identified contaminated sites in previous surveys. Reference sites, selected based on proximity to LHAAP and similarity to LHAAP water bodies, were not found to contain detectable levels of perchlorate. Each sampling location was labeled with its whole name or a 4-letter abbreviation. To date, five contaminated sites have been identified. Their names (and 4 letter abbreviations) are Harrison Bayou "catfish pond" (HBCP), Harrison Bayou upstream (HBUP), Goose Prairie Creek (GPRC), holding pond (HOLP), and a ditch by the old fire station (FSTC). There have also been four reference sites identified: Central Creek (CENC), Star Pond (STAR), Haggerty Creek (HAGC) and Karnack Creek (KARC). All names and abbreviations were recorded on data sheets and sample tracking forms and/or in the field notebook for future reference.

13. EXPERIMENTAL DESIGN INCLUDING BIAS CONTROL

13.1 Uptake of perchlorate

Fish or tadpoles were placed in minnow traps in contaminated and reference locations. The organisms were exposed concurrently, not having different exposure experiments: i.e., a group of animals were exposed and one third was removed from the cage on day 1, another third was removed from the cage on day 5 and the remaining third was removed on day 10. After removal, they were weighed, labeled, frozen, and transported back to the laboratory for perchlorate analysis. The placement of the cages was dictated by the physical structure of the stream or pond, and when possible, they were not deployed closer than 0.5 m apart. A minimum of 5 g of tissue is needed for perchlorate analysis, so the number of animals in each cage was adjusted to accommodate this amount of tissue, plus an additional amount of animals to account for at least 25% mortality. There were 1-5 cages deployed at each

site, depending upon the physical characteristics of the stream.

13.2 Reproductive effects on mosquitofish

Mosquitofish were placed in minnow traps (described above) in reference and contaminated sites. The standard length was measured (in mm) after being removed from the cages. The placement of cages was as described in 13.1. They were allowed to remain in situ for 6 weeks, or as long as possible. There were 10-20 fish per cage, and 1-5 cages per stream, depending upon the physical characteristics of the stream. The fish were removed and euthanized according to SOP AQ-1-03. They were preserved in 70% ethanol, dissected in the laboratory, and the number of embryos in each female was determined by counting.

13.3 Developmental effects in tadpoles

Tadpoles were caged as described above using minnow traps (13.1 and 13.2). The tadpoles used were of a size large enough that they would not escape from the minnow traps (as determined by preliminary experiments). Each cage had equal numbers of tadpoles of a given developmental stage. The total body length, tail length, and length of hind limbs, if present, were measured prior to being placed in cages; but because this resulted in high mortalities, the rest of the tadpoles deployed were only weighed. They were allowed to remain in the cages for 4-6 weeks. After this time, the length of the body, tail and hind limbs were recorded.

13.4 Bias control

Prior to deployment in cages, all animals (adult mosquitofish, mosquitofish fry, or tadpoles) were placed in one or more buckets. Each bucket contained only one species. They were then randomly assigned to each cage. Each cage was assigned a number from 1-12. Twelve buckets were then set up and numbered 1-12. A random number generator was used to randomly order the numbers 1-12, and this was done 5 times: e.g., 3-4-10-1-2-12-11-5-7-6-8-9, 8-4-12-7-10-6-9-5-1-2-3-11, 5-11-12-6-9-7-4-3-8-1-10-2, 12-4-9-3-5-7-1-10-2-8-11-6, 11-3-4-9-5-8-12-10-1-2-6-7. The animals were placed into the cages according to this list of numbers. For example, one fish or tadpole was placed into cage 3, then cage 4, cage 10, cage 1, cage 2, cage 12, cage 11, cage 5, cage 7, cage 6, cage 8 and finally cage 9. A second fish or tadpole was placed in each cage in the order 4-12-7-10-6-9-5-1-2-3-11, and so on, until there were 5 animals in each cage. If all 12 cages were not deployed in one day, then each day of deployment was treated as a block. The cages were

then split up evenly among blocks, and each site was represented in each block an equal number of times (i.e., the same number of cages was deployed at each site on any given day). The randomization procedure within each block followed the above scheme. A pair of dice was used for this purpose. Attaching one end of a piece of fluorescent twine to the cage and the other end to a stake or tree branch on the shore indicated the location of each cage. Different buckets were used for each site and buckets were washed between sampling events. Before taking previously used buckets into the field, buckets were washed with detergent (Alconox or other equivalent laboratory detergent) and tap water, and rinsed with tap water or other water known to be uncontaminated with perchlorate or other toxicants. Because detergent residues are toxic to larval amphibians, washing was done with water only if tadpoles were being used (perchlorate is fully soluble in water). The bucket was then rinsed with tap water 3 times. If mud, algae, or other residues remained on the inside of the bucket, it was scrubbed off during washing (a different brush was used for each sampling site). Reference sites were chosen so as to be as similar as possible to the contaminated site(s) in terms of habitat structure and stream characteristics. The area of pond/lake (m^2) or length of stream or lake shore (m) sampled at each site should be as similar as possible between sampling sites. The length of time animals were held in buckets was minimized, and was as similar as possible for all sites. The same species was collected from contaminated and reference sites. Animals were weighed prior to processing and weight was recorded on sampling form.

14. METHODS

14.1 Test System acquisition, quarantine, acclimation

14.1.1 Water Sampling

At each location where cages were deployed, 20 ml of water were also taken for perchlorate analysis, according to SOP AQ-3-03. Water was collected before deploying cages, whenever samples were removed for analysis, and at least once per week during exposures lasting longer than 1 week. Water samples were collected in precleaned glass vials (Wheaton), and were collected from just under the water surface. Water samples were stored away from direct sunlight and excessive heat (> 50° C). Before samples were taken, the pH, dissolved oxygen, conductivity, and temperature were measured according to SOP IN-2-01 and recorded on TIEHH form 181.

14.1.2 Fish Collection

Because mosquitofish are not susceptible to electroshocking, they

were collected by seining. The seine were at least 20' long and 4' deep, with a mesh of ¼" or smaller. Smaller seines may have been used if seining smaller water bodies. Any captured fish were placed in plastic buckets with aeration until processing. A different bucket was used for each site, or the buckets were cleaned as described in section 13.4.

14.1.3 Amphibian Collection

Larval amphibians were collected with dip nets and seines according to SOP AQ-3-05. Amphibians were also purchased from commercial suppliers as eggs or as developing tadpoles. If they were purchased as eggs, they were allowed to hatch in the laboratory and grow to at least 5 mm in length before being deployed in the field. If tadpoles were collected from the field, a subsample was preserved in 10% formalin and taken back to the laboratory for positive identification.

14.1.4 Caging

Plastic minnow traps (17 in. long x 9 in. wide at largest diameter with 3/16 in. (4.8 mm) square mesh for minnow-size fish. 7/8 in. (22 mm) diameter entrance hole were used for perchlorate uptake, reproductive and developmental effects. They were plugged at both ends with rubber stoppers large enough to totally occlude the opening. Some minnow traps were also covered with screen to avoid predators entering the cage. They were placed in water deep enough to submerge the traps by at least 50%.
Animals in cages were fed at least every other day, at a rate of approximately 0.1 mg food per gram of fish or tadpoles in the cage. Fish were fed tubifex worms and pelleted aquarium food such as shrimp pellets. Tadpoles were fed tadpole food or herbivore fish pellets supplemented with lettuce and spinach, approximately 0.1 mg food per gram tadpole for each.

14.2 Test Material Application

Rates/concentrations: Concentrations were determined by laboratory analysis.
Frequency: Animals were exposed to perchlorate when they were contained within the cages, as described in section 14.1.
Route/Method of Application: Ingestion or absorption of perchlorate from water and natural food items.
Justification for Exposure Route: The animals were exposed to perchlorate in water and food items in their natural environment.

Exposure Verification: Water samples were collected for determination of perchlorate concentrations wherever biota samples were collected.

14.3 Test System Observation

At every location where animals were caged, the following environmental parameters were evaluated: water temperature, pH, salinity, dissolved oxygen, and conductivity. Mosquitofish and amphibian cages as well as the environmental conditions were monitored. The important features of the cage (i.e., whether the cage is containing the fish /amphibians, preventing entry of predators and competitors, remains resting on the sediment) and if repairs may be needed were evaluated weekly. At no less than once every two weeks, the following environmental parameters were evaluated: water temperature, pH, salinity, ammonium perchlorate concentration, and dissolved oxygen.

14.4 Animal Sacrifice and Sample Collections

14.4.1 Data Recording and Sample Labeling

Prior to processing any samples, they were given a unique ID number. Species and weight were recorded on sample collection/dissection forms, as well as tissues collected and method of preservation. Fish length was an optional parameter and was recorded. Fish weight was measured on a portable balance to the nearest gram (if the fish weighs 10 grams or more) or to the nearest 1/10 gram if it weighs less than 10 grams. Prior to use, the scale was calibrated according to SOP IN-4-01, "Field Scale Operations and Maintenance" and calibration was recorded on TIEHH form 60.

According to the SOP IN-3-02, the minimum information to be recorded on labels was the project number and unique ID. The unique ID contained enough information to be able to identify the species. Date of collection, species, and exposure location were included on the label. Pre-printed labels were used, but if they were used on samples to be frozen or chilled on ice, the project number and unique ID were also printed on the container with indelible ink, or protected with tape. The unique ID included the 4-letter abbreviation denoting the species. For mosquitofish, this was denoted as MOSQ, for tadpoles, it was TADP. Another hyphen and the sample number followed this. For example, MOSQ-1 was the unique ID for mosquitofish #1 from cage #2. Sample numbers were assigned in the order in which they were processed.

14.4.2 Perchlorate Analysis

Fish and tadpoles collected were anesthetized with an overdose of MS222 (0.5 g/L) and frozen in liquid nitrogen for perchlorate analysis. Alternatively, fish were chilled on wet ice until transport back to the laboratory. Individual fish were wrapped in aluminum foil, labeled prior to freezing or chilling, and logged in the collection notebook. Smaller fish were pooled into composite samples. Composite samples were placed into Ziploc freezer bags and stored on ice until transport back to the laboratory. At least 5 g of tissue is needed for perchlorate analysis; therefore fish smaller than this were composited.

After transport to the laboratory, samples were stored in the freezer (temperature -20° C or colder) until analysis. The samples were then analyzed for perchlorate according to SOP AC-2-15 "Extraction and Cleanup of Tissue Samples to be Analyzed for Perchlorate".

Water samples were also transported back to the laboratory for perchlorate analysis. Once in the laboratory, they were stored in a refrigerator (4° C) until analysis. They were then analyzed for perchlorate according to SOP AC-2-11 "Analysis of Perchlorate by IC". Frozen tissue samples were kept on site and periodically (at least monthly) were transported to the laboratory by ground transportation. Water samples were shipped by overnight courier.

14.5 Endpoint Analysis

Analysis of samples for perchlorate was according to SOP AC-2-11 "Analysis of Perchlorate by IC".

Developmental effects in the amphibians were assessed by comparing the ratio of tail length/body length or hindlimb length/body length between caging sites. Reproductive effects in mosquitofish were assessed by comparing the ratio of brood size/body length between caging sites. The females were dissected and the broods removed and counted before birth.

14.6 Statistical Methods

All data were checked for normality using the Shapiro-Wilk W test. Homogeneity of variances was checked using Bartlett's or Lavine's test. Comparisons between sites were accomplished by analysis of variance (ANOVA) for multiple mean comparisons. Correlation coefficients were used to determine if residue levels correlate with biomarkers, reproductive, and/or population data.

15. PROTOCOL CHANGES/REVISIONS

See attached change in study documentation forms.

16. RESULTS

Initial experiments with tadpoles utilized cricket frog (*Acris crepitans*) larvae, but they either escaped from the cages or were killed by odonate larvae (dragonfly, damselfly) that entered the cages. In another trial, mosquito netting was glued onto the traps in order to prevent tadpoles from escaping and/or predators from entering. However, none of these tadpoles survived more than 2 weeks, which may have been due to reduced water flow and resultant anoxic conditions caused by the small mesh. Subsequent experiments used bullfrog tadpoles (*Rana catesbaena*) because cricket frog tadpoles were no longer available at this time, and because the larger bullfrog tadpoles were less likely to escape from the cages and be eaten by the odonates. However, there was still heavy mortality in all cages, and some cages were damaged and moved by mammals (otters or raccoons) and a few were destroyed by alligators (as evidenced by crushed and empty cages with teeth marks on them). There were two trials with the bullfrog larvae, and only 2 sites had surviving tadpoles after a 4-week exposure (Central Creek and Goose Prairie Creek, Crocket Ave. bridge). However, the results of the metamorphosis assay were contrary to what was expected: although all tadpoles started out at roughly the same developmental stage (stage 12-15), all of the Goose Prairie Creek individuals had metamorphed into froglets, while the tadpoles from Central creek still possessed tails and their forelimbs had not yet emerged.

For the mosquitofish experiments, in one trial all the fish died in the cages within 3 weeks. In the second trial, only two sites had surviving individuals after 4 weeks. These were Goose Prairie Creek and the INF Holding Pond. The mean fecundity for these sites (± SD) was 0.24 ± 0.30 and 2.26 ± 0.80, respectively, and the difference was statistically significant ($p<0.001$, t-test). The percent of gravid females for Goose Prairie was 52%, and for the Holding Pond it was 100%.

The concentration of perchlorate was not detectable in the water of the reference sites (Table 1). Among the contaminated sites (Table 1), the highest water concentration of perchlorate was found in the Holding Pond, however, perchlorate was detected in only 13 out of 18 samples. In Fire Station Creek, only 2 samples of water out of 21 presented detectable levels of perchlorate. No detectable levels of perchlorate were detected in the water collected at Goose Prairie Crockett.

Regardless of the sites (reference or contaminated), perchlorate was not detected in the mosquitofish (Table 2). In contrast perchlorate was detected in tadpoles from control (Central Creek) and contaminated (Fire Station Creek) sites. However, in both location only two samples contained perchlorate.

17. DISCUSSION

The high mortality of the fish and frogs in the cages was probably a result of high

water temperatures and resultant low dissolved oxygen in east Texas water bodies during the summer. Any future studies should be started in early spring and be concluded by May or early June in order to avoid such mortality. However, there seems to be a large inter-site variability in mosquitofish fecundity and tadpole developmental rates, which may be affected by variables incidental and independent of perchlorate exposure, such as pH, water temperature, productivity of the water, or dissolved mineral composition. This introduces a high degree of environmental noise and may limit the utility of these assays for determination of reproductive and developmental effects of perchlorate exposure *in situ*. Water and biota samples from Central Creek are being analyzed further in order to determine if there was a source of perchlorate that was overlooked in previous analyses.

Table 1. Concentration of perchlorate (ppb) in water collected from different sites at LHAAP

Sites	Concentration (ppb)	Sample size
Star Pond	trace[a]	3
Central Creek	trace[a]	23
Goose Prairie Crockett	trace[a]	16
Smith's Lake	trace[a]	2
Fire Station Creek	7.57 ± 27.81	21
Holding Pond	369.36 ± 344.60	18

[a]Perchlorate was detected, but the concentration was not high enough to accurately quantify

Table 2: Concentration of perchlorate (ppb) in mosquitofish and tadpoles collected from different sites at LHAAP

Sites	Concentration (ppb)	Sample size
Mosquitofish		
Central Creek	trace[a]	14
Fire Station Creek	trace[a]	12
Goose Prairie Crockett	trace[a]	7
Holding Pond	trace[a]	2

Tadpoles		
Central Creek	53.83 ± 83.61	6
Fire Station Creek	74.56 ± 147.95	9

[a]Perchlorate was detected, but the concentration was not high enough to accurately quantify

18. STUDY RECORDS AND ARCHIVE

Study Records will be maintained at The Institute of Environmental and Human Health (TIEHH) Archive for a minimum of one year after study completion date.

19. REFERENCES

Manzon RG and Youson JH. 1997. Immunocytochemical and morphometric study of TSH, PRL, GH, and ACTH cells in Bufo arenarum larvae with inhibited thyroid function. Gen. Comp. Endocrinol. 98: 166-176.

Miranda, LA, Paz, DA, Dezi, RE and Pisano, A. 1996. Immunocytochemical and morphometric study of TSH, PRL, GH, and ACTH cells in Bufo arenarum larvae with inhibited thyroid function. Gen. Comp. Endocrinol. 98: 166-176.

20. APPENDICES

Study Protocol
Changes to Study Documentation
Changes to study 1
Changes to study 2
Study Phase Inspection Reports
Daily monitoring for reproductive effects on mosquitofish and field safety procedures
List of Standard Operating Procedures